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A Colorimetric Method for Determination of Sulfhydryl Groups in Tissue Homogenates by 1-(4-Chloromercuriphenylazo)-Naphthol-2. (17868)

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Recently Bennett and Yphantis(1) reported the synthesis of 1-(4-chloromercuriphenylazo)-naphthol-2, henceforth referred to as red -SH reagent, because it forms a red precipitate with compounds containing free -SH groups. While the reagent appeared to be suitable for histochemical demonstration of -SH compounds(2), its low molecular extinction coefficient limited its use as a colorimetric -SH reagent(3). We have found that the addition of a strong mineral acid greatly intensifies the light absorption of the -SH reagent, thus making it possible to develop a simple colorimetric -SH estimation in tissue homogenates.

Experimental. The method is based on the observation that when an aqueous solution (or suspension) of -SH compounds is shaken with an amyl acetate solution of the red -SH reagent, a red precipitate forms in the aqueous layer. The amount of precipitate is directly proportional to the amount of -SH groups and can be determined by centrifugation, washing it several times, redissolving it in concentrated sulfuric or hydrochloric acid, and reading the extinction in a colorimeter.

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1. Bennett, H. S., and Yphantis, D. A., *J. Am. Chem. Soc.*, 1948, v70, 3522.

2. Bennett, H. S., *Anat. Rec.*, 1948, v100, 640.

3. Bennett, H. S., personal communication.

It is more convenient, however, to determine colorimetrically the decrease in concentration of the dye in the amyl acetate supernatant. The amount of -SH groups present can be read from a calibration curve obtained by shaking the amyl acetate solution of the -SH reagent with known amounts of an -SH compound (glutathione).

Reagents. 1. 1-(4-chloromercuriphenylazo)-naphthol-2. Three mg of the finely pulverized dye, prepared as described elsewhere (1), are dissolved in 100 ml of amyl acetate with continuous stirring until most of it is in solution. Heating to facilitate solution should be avoided because this produces a solution which is unstable. After filtration, the amyl acetate solution may be stored for a long period in a dark, glass stoppered bottle at 4°C, without appreciable change in its reactivity toward -SH groups. When an aliquot of this golden brown solution is mixed with half its volume of concentrated hydrochloric acid and allowed to stand for 15 minutes, the light absorption of the dye increases and gives a reading (log Io/Ix) of 220-320 in the Klett-Summerson colorimeter with filter 54. Maximum absorption occurs at 520 mμ. The color is stable for several days. 2. Concentrated HCl. 3. Glutathione standard‡ solution (10-100 mg glutathione freshly dissolved in 100 ml of distilled water).

Procedure. Five ml samples of the amyl acetate solution of the red -SH reagent are measured into glass stoppered centrifuge tubes. The tissues to be analyzed are chilled in an ice bath immediately upon removal from the freshly killed animal and weighed rapidly. They are then homogenized in ice cold medium with glass homogenizers(4). The homogenates are diluted to a final tissue concentration of 5%. One-tenth and 0.2 ml (5 and 10 mg) are pipetted into the amyl acetate solution of the red -SH reagent. The centrifuge tubes are stoppered and mechanically shaken for 20 minutes with an oscillation of 250 per minute. In preliminary

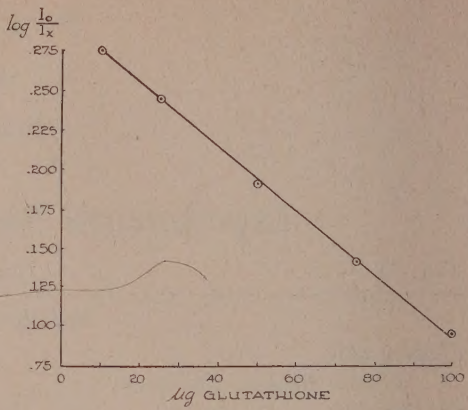

FIG. 1.
Calibration of –SH reagent with glutathione solution.

measurements 20 minutes' shaking was sufficient to complete the reaction between the -SH groups and the red -SH reagent. After centrifugation for 15 minutes at 2000 r.p.m., an aliquot of 4 ml of the clear amyl acetate supernatant is transferred to a test tube. Upon the addition of 2 ml of concentrated HCl, the red color develops immediately. The tubes are thoroughly shaken and allowed to stand for 15 minutes. The color is measured in the Klett-Summerson photoelectric colorimeter, with filter 54, against a blank containing 2 parts of amyl acetate and 1 part of HCl. An -SH standard curve is obtained by plotting the extinction of the acidified red -SH reagent against the amount of -SH groups (in the form of glutathione). Since the -SH reagent is insoluble in water, the values obtained are not measurably altered by the amount of water in which the tissue is homogenized or the glutathione dissolved. As a rule all tissue samples and -SH standards were made up to 0.5 ml with the homogenizing medium. In agreement with histochemical findings, the reaction of -SH groups with the red -SH reagent is completely abolished when iodoacetamide or mercuric chloride is added to the samples before the analysis.

Results and discussion. The apparent -SH content of tissue homogenates was found to depend on the nature of the homogenizing medium. Analyses of samples of the same mouse liver, homogenized in 0.9% NaCl,

‡ Obtained from Schwartz Laboratories, Inc., 202 E. 44th St., New York City.

4. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, v114, 495.

TABLE I.
–SH Content of Homogenates of Rabbit, Rat, and Mouse Livers (in γ/100 mg wet weight).

Liver of	Anson's method	Author's method
Rabbit	46.5	45.2
Rat	46.5	47.5
"	48.4	53.8
"	35.6	36.7
"	38.9	36.7
"	47.5	48.6
"	44.3	43.2
Mouse	34.5	35.6
"	31.3	36.5
"	52.9	59.4

Tissues were homogenized in 0.9% NaCl and diluted to a final tissue concentration of 0.5% for Anson's method and to 5% for the present method.

TABLE II.
–SH Content of Kidney, Heart, and Brain Homogenates (in γ/100 mg wet weight).

Tissue	Anson's method	Author's method
Rat kidney	25.2 (18.2-26.6)	56.4 (53.8-60.2)
" heart	20.1 (17.2-26.6)	48.9 (45.2-56.9)
" brain	15.6 (11.8-18.2)	59.2 (55.9-62.3)
Mouse kidney	20.4 (14.0-22.6)	54.3 (35.0-61.3)
" heart	17.2 (10.8-19.4)	41.9 (36.6-47.3)
" brain	14.0 (9.7-16.0)	52.7 (44.1-53.8)

Each figure represents the avg of determinations obtained in 6 animals. Min. and max. values in parentheses. Every determination carried out in duplicate on 2.5, 5, and 7.5 mg tissue samples with Anson's method and on 5 and 10 mg samples with the present method.

0.88 M sucrose, distilled H_2O, 10% urea (in H_2O), and 10% Duponol PC (Na-laurylsulphate), gave 29, 31, 47, 77, and 89 γ of -SH, respectively, per 100 mg of wet tissue. In all tissues analyzed, the lowest -SH values were obtained when NaCl or sucrose solutions were used as homogenizing media, an increase being noted with distilled H_2O or protein denaturing agents. The liberation of -SH radicals in protein denaturation is described by Anson(5).

Comparison showed acceptable agreement with results obtained by the ferricyanide procedure of Anson(6) on liver tissue (Table I), but higher values on kidney, brain, and heart (Table II). The discrepancy may be explained by the protein denaturing effect of amyl acetate on tissue proteins other than liver. The resistance of liver proteins to amyl acetate remains unexplained at present. Native egg white which showed no free -SH radicals with Anson's method(6) reacted with the red -SH reagent, indicating that protein denaturation had occurred during treatment with the amyl acetate solution.

The convenient procedure described in this paper offers the possibility of determining -SH groups in tissue homogenates. The optically clear solutions used for colorimetric measurements give it an advantage over procedures where the color of a turbid suspension has to be estimated. In further experiments, correlation between enzyme activities and -SH content of tissue homogenates is being investigated.

Summary. A colorimetric procedure was developed for the determination of -SH groups. The method involved the estimation of the amount of an organic mercury salt removed from its amyl acetate solution by shaking with tissue homogenates. The dye removed was found proportional to the -SH content of the sample analyzed.

5. Anson, M. L., *Adv. Protein Chem.*, 1945, v2, 361.

6. Anson, M. L., *J. Gen. Physiol.*, 1941, v24, 399.

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Hemolysis of Erythrocytes Incubated in Salines. (17869)

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The increase in fragility of erythrocytes caused by incubation of blood at 37°C for 12 to 32 hours, after which treatment the corpuscles may hemolyse even in serum or isotonic saline, has been shown to have differential value between bloods from normal

TABLE I.
Incubation Hemolysis of Dog Blood at 37°C.

	Initial osmotic hemolysis, 1 hr		Hemolysis due to incubation					
			5 hr		9 hr		22 hr	
% NaCl	.41	.70	.41	.70	.41	.70	.41	.70
M %	22*	n	3	6	14	23	47	77
t	—		1.67		2.53		4.12	
P	—		.10		.02		.001	
	—		n.s.		s.		v.s.	

M = Mean of 22 cases.
n = Negligible.
* Deducted from later readings of hemolysis in 0.41 NaCl.
n.s. = not significant.
s. = significant.
v.s. = very significant.

and hemolytic jaundice(1). This increase in fragility can be prevented largely by resuspending the corpuscles in fresh serum every 2 hours during the incubation period (1). In the present studies of hemolysis during incubation, 1:100 blood dilutions made up with salines of different osmotic tonicities were used to minimize effects of the serum. Within the gamut investigated it was found that the greater the tonicity of the saline the greater the incubation hemolysis.

Material and methods. Blood taken from dogs held under favorable conditions and fed standard rations was used. The blood was drawn under aseptic conditions. Quantities to make 1:100 dilutions were discharged at once into pyrex flasks containing saline solutions buffered to pH 7.35 with disodium phosphate(2) and held at 25°C. No anticoagulant was used. The sodium chloride was tested by the method of Ball(3) and found free from silver and other hemolytic substances. Five ml units of the diluted blood were pipetted at once into 30 ml pyrex test tubes which were stoppered with cotton plugs and placed in electrically controlled ($\pm 0.1°$ C) water baths. The tubes were not disturbed until they were removed for analysis. The experimental hemolysis and the total hemolysis were determined for each tube from readings made with a spectrophotometer set at 5450 Å U.

Results. The first group included 22 samples of dog blood, using both 0.41 and 0.70% sodium chloride solutions as the diluting fluids. All of the samples of blood tested showed some hemolysis (average 22%) during the first hour after dilution with 0.41% saline. As there was no significant difference in the amount of this initial hemolysis in series held at 25° and at 37°C, the hemolysis during the first hour was due apparently to the tonicity of the saline and was deducted from subsequent readings, to obtain the hemolysis due to incubation. The hemolysis during the first hour at 25° or 37°C was negligible in dilutions made with 0.70% or stronger salines.

In Table I the means of the hemolyses due to incubation at 37°C are presented, together with the significance of the differences between these means as determined by Fisher's "t" procedure(4). It can be seen that the incubation hemolysis in the 0.70% saline exceeded that in the 0.41% at the end of 5 hours and maintained that relation throughout the remainder of the incubation period. However, the slight difference at the end of 5 hours is not statistically significant and the individual data showed that there was much variation in the time of the onset of incubation hemolysis. By the ninth hour of incu-

1. Ham, T. H., and Castle, W. B., *Proc. Am. Phil. Soc.*, 1940, v82, 411.

2. Jacobs, M. H., and Parpart, A. K., *Biol. Bull.*, 1931, v60, 95.

3. Ball, E. G., *Biol. Bull.*, 1933, v64, 277.

4. Fisher, R. A., *Statistical Methods for Research Workers*, 4th ed., Edinburgh, Oliver and Boyd, 1932, 307.

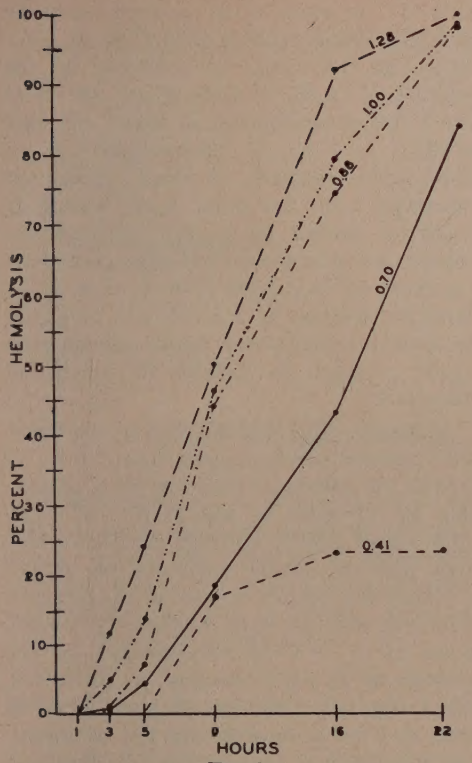

FIG. 1.

Mean % of hemolysis due to incubation at 37°C of 14 samples of dog blood in 0.41, 0.70, 0.88, 1.00, and 1.28% salines. The mean initial osmotic hemolysis at the end of the first hour, 37% has been deducted from the 0.41% series. There was no initial osmotic hemolysis in the other salines.

bation, however, the difference between the 0.41% and 0.70% series was definitely significant with P=0.02 and almost within the P=0.01 grouping. The difference between the 2 series is highly significant after 22 hours incubation, with P=0.001.

In 14 subsequent series of dog bloods the dilutions were made with 0.41, 0.70, 0.88, 1.00, 1.28% salines. The mean values are shown in Fig. 1. The hemolysis at the end of the first hour of incubation at 37°C in the 0.70 to 1.28% salines inclusive was negligible. From the second hour to the end of each incubation test the rate of hemolysis and after a time, the percents of incubation hemolysis and finally the observed hemolysis (incubation hemolysis plus initial first hour hemolysis) in the 0.41% saline were exceeded by the hemolysis in all of the salines of greater tonicity. In addition, the hemolysis in each of the several salines having negligible hemolysis during the first hour, in turn exceeded the hemolysis in all of the salines of lower tonicity.

Discussion. Ham and Castle(1) found that mammalian erythrocytes during incubation at 37°C exhibited pre-hemolytic sphering and it has been stated repeatedly(1,5,6,7) that the more nearly spherical the erythrocytes the greater their susceptibility to hemolysis. As the lower the osmotic tonicity of the saline the more complete the sphering of the corpuscles unless some other factor is superimposed, on the basis of osmotic relations alone it might seem that the hemolysis in the incubated series would vary inversely with the tonicity of the saline. However, in the present incubation series hemolysis varied directly with the percent of the saline. The lower incubation hemolysis in 0.41% saline might be thought due to the smaller number of corpuscles present after the first hour because of the initial osmotic hemolysis, or to a possible protective action of products of the initial hemolysis. When the hemolysis was computed for the 0.41% and 0.70% series after 22 hours incubation for all of the 36 samples, on the basis of number of corpuscles present in the 0.41% series after the initial osmotic hemolysis, the mean hemolysis for the 0.70% series was over 17% greater. This value was highly significant by the "t" procedure in spite of the facts that an average of 22% of the corpuscles were eliminated from the 0.41% series by the initial osmotic hemolysis, and that the 0.70% series were approaching total hemolysis after 22 hours incubation. Some subsidiary tests in which hemolysed erythrocytes were added to preparations having no initial hemolysis and others in which the prod-

5. Hayden, R. L., *Am. J. Med. Sci.*, 1934, v188, 441.

6. Castle, W. B., and Daland, G. A., *Arch. Int. Med.*, 1937, v60, 949.

7. Ponder, Eric, *Hemolysis and Related Phenomena*, Grune and Stratton, 1948, 102.

ucts of the initial hemolysis were removed from the corpuscles remaining before these were incubated showed that the products of hemolysis had little or no influence on the development of incubation hemolysis in these series. As there was no initial osmotic hemolysis in all of the series including and above 0.70% saline direct comparisons can be made between those series. The possible effect of the phosphate buffer on hemolysis was eliminated by substituting a carbonate buffer in some of the tests. The results were essentially the same with the two buffers. Ham and Castle(1) infer that the increases in erythrocytic volume and fragility during incubation are due to metabolic processes which produce an increase in the osmotically active constituents of the erythrocytes. If this explanation be accepted it would seem that the salines from 0.41% to 1.28% are progressively more favorable to these postulated metabolic changes. The nature of these metabolic changes has not been determined, but Harris(8) followed the losses and gains in potassium and sodium by human erythrocytes with and without dextrose and concluded that metabolic activity was responsible for the movement of these cations.

In the present experiments it was found that 95% or more of the expected hemolysis in 0.70% saline resulting from 22 hours incubation at 37°C could be prevented by the addition of 32 mg of dextrose per 100 ml of the 1:100 saline dilutions of blood, and that as little as 1 mg of dextrose per 100 ml appreciably reduced 22 hour incubation hemolysis. On the other hand, hemolysis could be speeded up greatly, although the relative action of the several salines remained the same, by incubation at 42°C or at 45°C. Both the reaction to dextrose and to higher incubation temperatures might suggest metabolic changes as factors in incubation hemolysis.

Summary. In 1:100 dilutions of dog blood with buffered salines ranging from 0.41% to 1.28% the hemolysis resulting from incubation for 22 hours or less at 37°, 42° and 45°C varied directly although not proportionately with the osmotic tonicity of the saline.

Ninety-five percent or more of the expected hemolysis resulting from incubation in 0.70% saline at 37°C could be prevented by the addition of 32 mg of dextrose to 100 ml of the 1:100 saline dilution of blood, and as little as 1 mg of dextrose per 100 ml appreciably reduced 22 hour incubation hemolysis.

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8. Harris, J. E., *J. Biol. Chem.*, 1941, v141, 579.

Effects on Anaphylactic Shock of Salicylates, Aminopyrine and Other Chemically and Pharmacologically Related Compounds.* (17870)

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The effect of salicylates on antibody formation and antigen-antibody reactions has been studied sporadically for many years(1). Recently, Campbell(2) prevented anaphylaxis in a small number of animals by the administration of aspirin. Because salicylates have had a long, useful history in medical therapy, it seemed highly desirable to extend Campbell's results to learn more about the phenomenon and also to search for related

* Aided by a grant from the Institute for the Study of Analgesic and Sedative Drugs.

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1. Smith, P. K., *J. Pharmacol.*, Part 2, 1949, v97, 353.

2. Campbell, B., *Science*, 1948, v108, 478.

TABLE I.
Effect of Various Compounds on Number of Deaths from Anaphylactic Shock.

Compound	Dose	Control D/T*	Control % deaths	With compound D/T*	With compound % deaths	Chi²
Acetylsalicylic acid	.64 g	39/74	52.8	8/57	14	21.3
Sodium salicylate	.57	32/60	53.3	9/43	20.9	11.0
Aminopyrine	.82	19/34	55.9	1/22	4.5	14.5
Acetanilid	.48	27/52	51.9	15/42	35.7	2.4
Neocinchophen	1.04	15/35	43	10/28	35.7	0.4
Sodium para-aminobenzoate	.56	18/43	41.8	9/34	26.4	1.9
Para-aminosalicylic acid	.54	19/42	45.2	9/31	29	2.0
Ortho-aminobenzoic acid	.49	8/17	47	4/18	22.2	2.4
Para-hydroxybenzoic acid	.49	8/17	47	4/18	22.2	2.4
Sulfanilamide	.61	5/15	33.3	2/16	12.5	2.0
Sodium gentisate	.63	8/28	28.5	8/19	42	1.1
" bicarbonate	.32	13/32	40.6	16/32	50	0.6
Nembutal	30 mg	14/28	50	11/30	36.7	1.1
Codeine	5	5/9	55.5	4/7	57.2	—

* Ratio deaths to total number of animals.

compounds with perhaps greater activity. As a first step, we have attempted to ascertain whether or not the inhibition by salicylates is related to (1) a particular type of chemical configuration, or (2) a specific type of pharmacological activity.

Method. Young rabbits weighing approximately 2 kg were sensitized by repeated injections of the undiluted white of hens' eggs. In the first 2 experiments, one with aspirin and one with nembutal, 3 injections of 1.0 cc were given on alternate days. The first 2 injections were given intravenously and the third, intramuscularly. In an attempt to increase the percentage death rate from anaphylactic shock, the animals in all later experiments received 2 additional subcutaneous injections of 1.0 cc on alternate days, a total of 5 injections over a 10-day period. Two weeks were allowed to elapse after the last injection for the development of sensitivity to the egg white. The animals were then shocked by 2 cc of undiluted egg white, administered intravenously.

For each new experiment, a separate group of control animals was studied. This was necessary because of the rather large variation in death rate among the control groups. An attempt was made to grade the degree of shock in the animals, but this proved to be too subjective. Consequently, in the preparation of our data, *only death rates were employed.*

The protective effect of a compound against anaphylactic shock was investigated as follows: sensitized animals were divided into an experimental and a control group. Approximately one hour before the shocking dose of egg white, the experimental group received the calculated amount of the compound suspended in water, and the control group, an equivalent amount of water, by intrapharyngeal tube. Thus, the 2 groups received equivalent handling and fluid intake. One hour after medication, the animals were shocked by intravenous administration of egg white. Most of the drugs were administered orally in equimolar doses but the nembutal and codeine were given subcutaneously in smaller doses. Neocinchophen was difficult to administer by the tube method because of its insolubility; therefore, we are not sure of the exact dosage in this group of animals. Also, because this compound is absorbed less readily than the others, an extra dose was given 10 hours before injection of the shocking dose of egg albumin. As an added precaution, 2 to 3 hours were allowed to elapse between administration of the final dose of drug and the shocking dose of egg albumin.

Blood salicylate levels were determined by the method of Brodie *et al.*(3) in rabbits of the same weight one hour after administration of doses similar to those given above.

Several experiments were conducted to de-

3. Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharmacol.*, 1944, v80, 114.

TABLE II.
Number of Experiments with Each Compound and Reproducibility of Results.

Drug	No. of experiments			
	Treated animals showed fewer deaths than simultaneous controls	Controls showed fewer deaths than treated animals	Treated = control	Total
Acetylsalicylic acid	7	0	1	8
Sodium salicylate	7	0	0	7
Aminopyrine	4	0	0	4
Acetanilid	3	3	0	6
Neocinchophen	2	1	1	4
Sodium para-aminobenzoate	2	1	2	5
Para-aminosalicylic acid	3	1	1	5
Ortho-aminobenzoic acid	2	0	1	3
Para-hydroxybenzoic acid	2	0	1	3
Sulfanilamide	2	1	0	3
Sodium gentisate	0	2	0	2
" bicarbonate	1	4	0	5
Nembutal	2	3	0	5
Codeine	0	1	0	1

termine the antihistaminic effect of the active compounds. Four mg of histamine hydrochloride in 1 cc 0.85 percent NaCl injected intravenously, killed about 80% of a group of rabbits. This dose was therefore used as a standard shocking dose. Animals were divided into 2 groups: the control group received water by intrapharyngeal tube, and the experimental group, the compound, suspended in water, one hour before the histamine shock dose.

Results. Both acetylsalicylic acid and sodium salicylate prevented death under the conditions of the experiment (Table I). Aminopyrine was also effective. We were not able to demonstrate a definite effect with any of the other compounds.

Table II indicates the degree of reproducibility observed in separate experiments with the various compounds. Again it can be seen that the death rates among animals receiving salicylates and aminopyrine were consistently lower than in the corresponding control groups. Thus, in 7 of 8 experiments with acetylsalicylic acid, in 7 of 7 experiments with sodium salicylate, and in 4 out of 4 experiments with aminopyrine, the animals were protected. No such clear-cut effects were observed with any of the other compounds. In the doses studied, the serum salicylate levels in rabbit plasma following administration of aspirin ranged from 11.5 to 20.0 mg % and those for sodium salicylate, 18.0 to 26.3 mg %.

An attempt was made to determine whether the antipyretic effect of the compounds was related to the anti-anaphylactic activity. Aminopyrine was the most effective antipyretic compound, reducing the temperature 3 to 4°F. Acetanilid and neocinchophen were almost as effective. Salicylates were less effective, lowering the temperature only 1 to 2°F. Even in individual animals, there was no correlation between the degree of antipyresis and anti-anaphylaxis. Thus 2 salicylate animals with the most marked temperature response died, whereas others with a minimal response tolerated the shocking dose of albumin.

Both aspirin and aminopyrine were tested for antihistaminic activity. Of 10 animals receiving aspirin, 9 died in histamine shock, compared with 7 deaths among 10 controls. With aminopyrine, there were 6 deaths among 9 animals receiving the compound, and 7 deaths in 9 control animals.

Discussion. Some previous studies, in addition to those of Campbell(2), indicate that salicylates may suppress the union of antigen and antibodies. Thus Coburn and Kapp (4) found that salicylates inhibited the pre-

4. Coburn, A. F., and Kapp, E. M., *J. Exp. Med.*, 1943, v77, 173.

cipitation of horse serum euglobulin by rabbit antiserum, and also decreased the amount of precipitate in a system composed of crystalline egg albumin and its antibody. Moreover, precipitates previously formed could be partially dissolved. The effect was proportional to the salicylate concentration. They attribute the effect to "inactivation of the antibody" rather than to suppression of antigen-antibody combination. It seems likely that salicylates also inhibit the formation of antibodies. Swift showed that the production of antibodies in response to various streptococcic and pneumococcic antigens could be suppressed by the administration of sodium salicylate during the period of antibody development(5). Derick and co-workers(6) showed that when salicylates and neocinchophen were given within 24 to 48 hours and continued for 10 to 14 days after serum administration, they suppressed the arthritis as well as the antibody response in man. In both studies the sera tested for antibodies were not free from salicylates at the time of the determination.

The production of anti rH agglutinins in guinea pigs and rabbits was reduced by sodium salicylate administration, according to Homburger(7). At the time of the test, no free salicylates were present in the serum. Scherer, however, was unable to confirm this finding in rabbits(8). It was shown by Jager (9) that a salicylate serum level of 30 to 40 mg % reduced the antibody response to typhoid antigen. Salicylate effects in streptococcal infections are not so clear-cut. Thus, Rantz, *et al.*(10) found no effect on the antistreptolysin and antifibrinolysin titers; according to Perry(11), however, antifibrinolysin titers are lower after salicylate therapy. Salicylates can frequently prevent or mitigate the endocardial lesions which follow hypersensitization of rabbits, according to Smull and co-workers(12) and Roberts *et al.* (13). Sullivan(14) and Smull *et al.*(12) reported decreased occurrence of arterial lesions in animals similarly treated, but this observation was not confirmed by Roberts *et al.* (13) and Forman and co-workers(15). Good, Campbell and Good(16) studied the encephalitis produced in guinea pigs by administration of guinea pig brain plus special adjuvants. They were able to prevent the encephalitis either by a combination of moderate amounts of salicylates and para-aminobenzoic acid, or by large amounts of salicylates alone. Aminopyrine and neocinchophen also have been said to inhibit antibody formation(6,17).

Our experiments indicate that both salicylates and aminopyrine are effective in preventing death from anaphylactic shock in rabbits. The drug was administered only once, one hour before the animal was shocked. Therefore, the effect cannot be attributed to inhibition of antibody formation. The results we have obtained confirm those of Campbell's(2) for salicylate but are based on a larger number of animals, and a more rigorous criterion of activity, *i.e.* death of the animal *versus* survival, instead of the grading of symptoms. We present a new observation, namely, that aminopyrine also inhibits anaphylaxis. Our results suggest that salicylates and aminopyrine affect the immune mechanism by interfering with the specific interaction of antigen and antibody. For example, death in animals receiving his-

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tamine is quite similar to death from anaphylactic shock. But one would have expected a reduction in the number of histamine deaths if the compounds under study produced merely a non-specific increase in resistance. Furthermore, no correlation could be found between anti-pyretic and anti-anaphylactic activity. Also, a different type of analgesic, codeine, and the hypnotic compound, nembutal, were ineffective. However, it is impossible to state with certainty that pharmacological or chemical effects other than interference with the immune reaction may not be important.

The "negative" results obtained with the other compounds indicate only that they are not as effective as salicylate and aminopyrine. One cannot be sure that more tests with this or other methods will find them devoid of activity. The results with neocinchophen may have been influenced by difficulty in administration and absorption. However, because this compound produced a definite anti-pyretic effect, it appears certain that a fairly large percentage of the dose must have been absorbed.

The failure of sodium gentisate to prevent anaphylactic shock is interesting because of the suggestion of Meyer and Ragan(18) that salicylate acts in rheumatic fever only after conversion to gentisate.

It is hoped that further studies with salicylates, aminopyrine, and related compounds will elucidate the site and mechanism of action of the inhibitory effect. Their influence on other protein, as well as non-protein, sensitizations should be of interest. A simpler technic for screening compounds for activity is desirable because the present method is too cumbersome and expensive.

Summary. 1. Oral administration of salicylates and aminopyrine in rabbits decreased the incidence of death from anaphylactic shock. 2. A number of compounds closely related, chemically or pharmacologically, did not produce a similar inhibitory effect. 3. The mechanism of action is not antihistaminic, nor does it appear to be related to characteristic pharmacological effects, *e.g.* antipyresis. It may represent a direct effect on the specific combination of antigen and antibody.

We are indebted to Dr. A. M. Griffin, The George Washington University School of Medicine, and to Dr. Jules Freund, the New York Public Health Institute, for advice.

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Effect of Metal-Combining Globulin (Fraction IV-7) in Severe Mediterranean Anemia.* (17871)

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Iron is transported in the serum only in attachment to a B_1-globulin, called the "metal-combining globulin", which resides in Fraction IV-7 of the plasma(1). Except under certain conditions this globulin is not completely saturated with iron, and the excess in reserve is measurable. The determination of the serum iron level measures the degree of saturation of this protein. The method of Rath and Finch(2) employed for the determination

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

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2. Rath, C. E., and Finch, C. A., *J. Clin. Invest.*, 1949, v28, 79.

TABLE I.
Effect of Injection of Metal-Combining Globulin (Fraction IV-7) in Severe Mediterranean Anemia.

Patient	Age yr.	Fraction IV-7 inj., g	Interval after infusion hr	Interval after infusion min.	Serum Fe content, γ/100 ml	Latent Fe binding capacity serum	Hemoglobin, g	Hematocrit, %
S.	11½	4		0	134.1	0	6.3	24.4
				5	142.7	75	6.6	20.4
				20	141.1	150	6.6	20.2
			1	5	193.3	0	8.2	21.0
			2	5	190.7	50	6.9	20.6
			4	15	169.0	0	7.3	22.3
			24		224.0	100	7.1	22.5
			48		204.6	0	7.3	20.5
				Splenectomy* 12/1/49				
		5		0	227.8	0		
				5	210.9	120	12.0	36.
				35	217.2	150	13.1	40.
			1	5	248.7	167	13.6	41.
			2	5	212.4	155	13.3	42.5
			3	5	240.8	0	13.1	39.
			5	5	229.3	0	13.1	40.5
I.	15	5		0	291.4	0	8.0	26.
				30	305.1	0	8.3	30.
			1		334.1	0	8.7	26.5
			2		327.0	0	8.6	27.3
			4		322.0	0	8.5	27.0
A.V.	12	5		0	167.1	0	8.0	26.5
				15	243.9	100	6.2	22.0
				45	211.0	50	6.9	26.0
			1	15	244.2	0	7.4	26.0
			2	15	237.8	0	7.5	24.5
			4	15	226.4	0	7.0	23.5
A.	13	10		0	207.7	0	8.0	24.5
				8	283.1	150	6.2	20.2
				33	402.8	0	7.4	24.0
			1	38	391.6	0	7.0	22.3
			2	38	341.0	0	6.4	21.0
			5	15	328.9	0	7.4	23.0
			24		195.5	0	7.7	26.5

* Between 11/25 and 11/29/49, 5 transfusions, totaling 1,250 cc of blood were given. This accounts in large measure for the elevated blood levels of the post-splenectomy period.

of the reserve, latent or unbound iron capacity of the serum depends on the addition of known quantities of iron to measured amounts of serum with the formation of a distinctive color-complex when iron is linked to the metal-combining globulin. The total iron binding capacity is the sum of the two values (serum iron level and latent iron-binding capacity). In a previous study(3) the blood of children with severe Mediterranean anemia was found to have an elevated serum iron and no latent or reserve iron-binding capacity. In normal children and adults, by contrast, the iron-binding capacity is quantitatively higher and the serum iron levels comprise approximately one-third of the total capacity. Comparable changes were noted in children with spherocytic anemia and sickle cell anemia. The marked reduction in total iron-binding capacity in these related hemolytic anemias suggested a congenital and possible transmissable deficiency of the metal-combining protein. With the availability of this plasma component in purified form†, tests were made to determine

3. Smith, C. H., Sisson, T. R. C., Floyd, W. H., Jr., and Siegal, S., *Pediatrics*, 1950, v5, 799.

† For furnishing this material and for many helpful suggestions, we are indebted to Dr. Douglas M. Surgenor, Harvard Medical School, Boston, Mass.

whether the serum iron and iron-binding capacity could be altered by its administration. Four children with the severe form of Mediterranean anemia were the subjects of the present investigation. Their blood was characterized by an elevated serum iron and no capacity to further bind iron. In one of these patients (S) the effect of administering the Fraction IV-7, containing the metal-combining globulin, was observed before and after splenectomy. Immediately preceding and at intervals following the intravenous injection of Fraction IV-7 over a period of ½ to 1 hour in amounts varying from 4 to 10 g in 500 cc normal sodium chloride solution, blood samples were withdrawn for analysis as noted in Table I. Serum iron levels, the capacity of the serum to bind iron, hematocrit and hemoglobin were determined on each sample in duplicate. The methods for the first 3 measurements have been previously described(2,4,5). Hemoglobin was determined by the method of Evelyn(6). The measurement of the serum iron-binding capacity(2) depends on optical density; full capacity to combine with iron is indicated by no further changes in density except in later phases due to the factor of dilution. The end point is marked by the salmon-pink color produced when increasing amounts of iron are added to the metal-combining protein.

Results. In each subject the serum iron level rose, most strikingly in patient D who had received 10 g of the fraction. The magnitude and duration of the post-injection rise differed in each subject. In general, the use of 5 g and more of Fraction IV-7 produced a rise sustained for 3 to 5 hours. In patient S the level remained elevated for 24 hours after the administration of 4 g of the fraction, whereas after 10 g in patient D there was a return to the lower pre-injection level within the same period. The varied curves probably reflect the extreme individuality of each case of Mediterranean anemia in regard to the degree of hemolysis and resulting iron storage. The consistent increase in serum iron following the injection of the metal-combining globulin suggests the release of iron from the tissues to the blood. These observations are in contrast with changes occurring in chronic infection. In the latter, Cartwright and Wintrobe(7) noted that after the injection of the globulin the temporary restoration of the total iron-binding capacity to normal from initially reduced levels was not accompanied by mobilization of iron from storage depots.

In subject S, both before and after splenectomy, in V, and in D, the injection of Fraction IV-7 produced a transitory latent capacity to bind iron in the serum. The effect was prolonged in S after splenectomy. The fleeting, ill-sustained appearance and reappearance of a latent iron-binding capacity in S before splenectomy cannot be correlated with serum iron levels, and no explanation for it can be assigned at this time. Irregularity in the latent iron-binding capacity, as compared with the consistent alterations in serum iron levels, may be due either to physiologic changes or to errors inherent in the method. No latent capacity to bind iron was induced in subject C, whose initial serum iron level was higher than all the rest.‡ The greater magnitude of serum iron and iron-binding capacity in S following splenectomy may be attributed in part to the injection of 5 g of the fraction as compared with 4 g prior to the operation. While subject C with a high initial serum iron value, had a splenectomy in early childhood, the possible effect of the removal of the spleen on serum iron values awaits confirmation with more extended experience. The effect of administering Fraction IV-7 to children without anemia is under

4. Kitzes, G., Elvehjem, C. A., and Schuette, H. A., *J. Biol. Chem.*, 1944, v155, 653.

5. Smith, C. H., *Am. J. Dis. Child.*, 1948, v75, 505.

6. Evelyn, K. A., *J. Biol. Chem.*, 1936, v115, 63.

7. Cartwright, G. E., and Wintrobe, M. M., *J. Clin. Invest.*, 1949, v28, 86.

‡ Since this paper was written, the administration of 20 g of Fraction IV-7 to this subject has resulted in a greater elevation of the serum iron level and the brief appearance of a latent iron-binding capacity with maximum values of 617 γ and 167 γ/100 cc of serum respectively. Treatment with increased quantities of the fraction is being investigated in other cases.

investigation.

Comparable results were obtained in a child with severe sickle cell anemia. Upon the administration of 5 g of Fraction IV-7, the serum iron rose from 245γ/100 cc of serum to a maximum of 310 γ in approximately 4 hours. No latent iron-binding capacity appeared.

Whether Fraction IV-7 furnishes a factor required for hemoglobin synthesis and red cell formation could not be established with the amounts of this material employed in the present study and within the short observation periods. Table I, which provides figures for 24 hour periods of observation, reveals entirely insignificant deviations of the hemoglobin and hematocrit values from the pre-injection levels.

Conclusion. The administration of Fraction IV-7 of plasma to 4 patients with severe Mediterranean anemia resulted in a consistent rise in serum iron values in all, and in a transitory appearance of a latent capacity to further bind iron in 3 of the 4 subjects. These observations suggest the release of iron from the tissues to the blood when additional metal-combining globulin is supplied in this disease. Further studies are in progress to explore the clinical value of Fraction IV-7 both in Mediterranean anemia and exogenous hemochromatosis associated with multiple transfusions.

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Absorption and Excretion of Terramycin in Humans: Comparison with Aureomycin and Chloramphenicol.* (17872)

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Terramycin is a recently discovered antimicrobial compound which is produced by a species of Streptomyces(1). This drug has been found to be active against a number of bacterial species *in vitro* and to suppress experimental infections in mice(2). Clinical investigation of terramycin is now in progress (3). Studies in laboratory animals have indicated that terramycin, like aureomycin and chloramphenicol, is well absorbed following oral administration(4). It seemed desirable, therefore, to make a study of the absorption, distribution, and excretion of terramycin following its administration to humans and to compare the data with those obtained following the administration of aureomycin or chloramphenicol (Chloromycetin®). In addition, the serum concentrations of these drugs attained in patients under treatment with various dosage regimens have been determined.

Materials and methods. Preparations of drug. Terramycin‡ was supplied as the amphoteric base in 250 mg capsules and in 2.5 g vials for aqueous suspension and as the hydrochloride in 250 mg capsules and com-

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† Postdoctorate Research Fellow, National Institutes of Health, U. S. Public Health Service.

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2. Hobby, G. L., Dougherty, N., Lenert, T. F., Hudders, E., and Kiseluk, M., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 133.

3. Knight, V., to be published.

4. Hobby, G. L., Reed, W., Rinne, D., Powers, M., and D'Ambrosia, A., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 138.

‡ Supplied by Charles Pfizer and Co., Brooklyn, N.Y.

TABLE I.
Serum Concentrations of Terramycin Base and Hydrochloride in 28 Subjects After a Single Oral or Intramuscular Dose.

	Subj.	Dose, mg/kg	Serum conc. in μg/cc ½	1	2	4	6	24 hr
Terramycin base, oral	1	50		2.5	5	10	10	2.5
	2			2.5	250	4	4	<2.5
	3			<2.5	<2.5	<2.5	2.5	<2.5
	4			62	200	250	250	<2.5
	5			<2.5	<2.5	2.5	2.5	<2.5
	6	25		<2.5	<2.5	<2.5	2.5	<2.5
	7			<2.5	2.5	5	5	<2.5
	8			<2.5	2.5	2.5	2.5	<2.5
	9			<2.5	<2.5	<2.5	2.5	<2.5
	10			<2.5	2.5	2.5	2.5	<2.5
Terramycin hydrochloride, oral	11	50		<2.5	6.2	12	6.2	<2.5
	12			6.2	6.2	12	12	<2.5
	13			6.6	6.6	6.6	4	<2.5
	14			6.6	4	4	2.5	<2.5
	15			12	13	13	13	12
	16			6.6	3.3	3.3	2.5	<2.5
	17				3.3	3.3	2.5	
	18				5	5	4	
	19	25		6.2	6.2	12	12	3.1
	20			<2.5	<2.5	<2.5	<2.5	<2.5
	21			<2.5	6.2	6.2	6.2	<2.5
	22			<2.5	<2.5	<2.5	2.5	<2.5
	23			<2.5	3.3	3.3	3.3	<2.5
Terramycin base, I.M.	24	10	16	5	2.5		<2.5	<2.5
	25	10	5	5	5		<2.5	<2.5
	26	5	2.5	2.5	2.5		2.5	<2.5
	27	5	5	5	5		<2.5	<2.5
	28	5	5	5	5		2.5	<2.5

N.B. 2.5 μg/cc = minimal measurable concentration.

pressed tablets. Aureomycin hydrochloride§ was available in 250 mg capsules and in 50 mg vials. Chloramphenicol‖ was provided in 250 mg capsules.

Collection of specimens. In the studies of absorption and excretion following oral administration, all subjects were fasted for 12 hours preceding the ingestion of a single large dose of drug. Blood specimens were drawn with sterile precautions at intervals after the drug administration. The urine was likewise collected at intervals during the 8 or 24 hour period following the ingestion of drug, all specimens were pooled, and aliquots were sterilized by Seitz filtration prior to assay. Specimens of serum and other body fluids were collected at random from patients receiving daily maintenance doses of these drugs. Simultaneous withdrawals of blood and cerebrospinal fluid were made from a number of patients who had been given a single large dose of drug several hours before lumbar puncture or from patients receiving maintenance doses. All serum, urine, spinal fluid, and other specimens were kept frozen until time of assay.

Assay technic. Microbiologic assay was carried out both with a 2-fold serial dilution technic and a modification thereof which provided fractional dilutions of the unknown. In the latter technic(5) after a preliminary 1:4 dilution of the unknown serum in broth,

§ Supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.

‖ Supplied as Chloromycetin® by Parke, Davis and Co., Detroit, Mich.

5. Tompsett, R., Shultz, S., and McDermott, W., *J. Bact.*, 1947, v53, 581.

subsequent dilutions were made in 25% pooled serum broth. When the broth inoculum of culture had been added, the final concentration of serum in each tube became 20%. The final dilutions of the unknown were 1:5, 1:6.6, 1:8, 1:10, etc. This method obviated the effect which varying concentrations of serum proteins may exert upon such an assay procedure. For non-protein fluids such as cerebrospinal fluid, a preliminary 1:4 dilution was not made. The final dilutions of these specimens were 8:10, 7:10, and 6:10 in the first 3 tubes of the series respectively. The test organisms used in the assay procedures were *Bacillus cereus*¶ and *Streptococcus hemolyticus*, C203Mv. The tests were read after 18 to 24 hours incubation at 37°C. Standard solutions of the drugs for which the unknown specimens were being assayed were diluted, inoculated, and incubated concurrently with each series of unknowns.

Results. Absorption and excretion. A single oral or intramuscular dose of terramycin base or hydrochloride was administered to 28 subjects. The largest single doses given were 4 g by mouth and 0.6 g intramuscularly. Intramuscular injections of the base were irritating, so that only a few determinations were made with this preparation by this route. The serum concentrations obtained in this study are presented in Table I. The maximum concentrations were attained usually within 2 hours after an oral dose and within 30 minutes after intramuscular injection. These were of the order of 12 to 16 μg per ml after a dose of 50 mg per kilo by mouth or 10 mg per kilo intramuscularly. Two elderly persons attained serum concentrations of 250 μg per ml after an oral dose of 2.5 g of terramycin base. These were the highest serum concentrations measured and were out of proportion to the other serum determinations. Although renal function was not investigated in these 2 subjects, it is possible there may have been impairment of drug excretion. The median serum concentrations after oral administration of terramycin base and hydrochloride are shown in

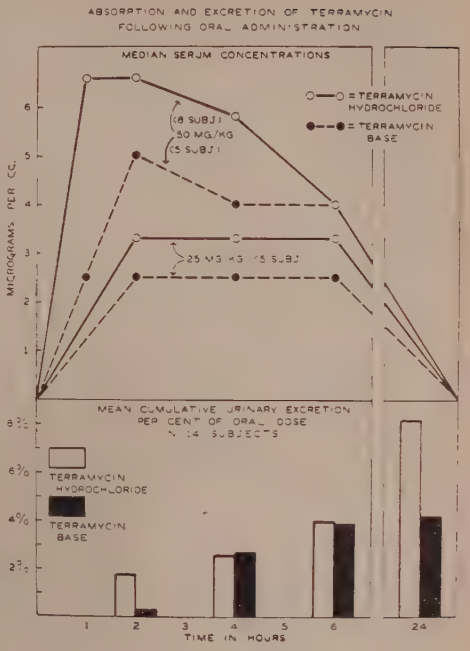

FIG. 1.

Fig. 1. The differences between the 2 preparations were not statistically significant ($p > 0.05$). Data for the urinary excretion of terramycin in 14 subjects are presented in Table II. The mean values for the amount of drug recovered in biologically active form in the urine after oral administration are shown in Fig. 1. These differences were likewise not significant ($p > 0.20$). Six adult males received a single oral dose of 50 mg per kilo of chloramphenicol; 3 days later the same 6 subjects were given an equal dose of aureomycin. The individual doses ranged from 3.25 to 4.25 g. The serum concentrations of these drugs are presented in Table III, and the median concentrations are shown in Fig. 2. Data concerning the urinary excretion are presented in Table IV. The mean cumulative excretion of the 2 drugs in biologically active form is shown in Fig. 2. The serum concentrations and the urinary excretion of chloramphenicol in biologically active form were consistently and significantly higher than those of aureomycin ($p < 0.01$).

Serum concentrations during maintenance

¶ This strain was obtained from Mr. A. C. Dornbush, Lederle Laboratories.

TABLE II.
Urinary Excretion of Terramycin Base and Hydrochloride After Oral and Intramuscular Administration in 14 Subjects.

	Subj.	Dose, mg/kg	Urine conc. μg/cc 0-2	2-4	4-6	6-24 hr	% dose recovered 2	4	6	24 hr
Terramycin, base oral	1	50	26	1790	102		.5	5.9	7.0	
	2	25	1.6	6.4	6.4	13	.4	3.2	5.4	6.5
	3	25	.2	51	51	102	.0	1.1	1.4	4.5
	4	25	6.4	13	13	6.4	.2	.8	1.2	1.8
Terramycin hydrochloride, oral	5	50	26	819	205	205	1.1	3.3	5.0	13
	6	50	26	103	103	205	.4	1.5	2.4	6.8
	7	25	2500	410	102	1250	4.7	7.8	10.6	26
	8	25	<.4	.8	13	51	.0	.0	.3	1.7
	9	25	26	410	410	625	.8	1.0	1.6	3.4
Terramycin, base intramuscular	10	10	22				.2			
	11	10	2.8		45*	11	.1		1.3	3.7
	12	5	.4		.8*	6.4	.0		.1	.4
	13	5	.4		<.4*	6.4	.0		.1	1.3
	14	5	410		26*	.8	10.8		11.3	11.6

* 2-6 hr grouped urine.

TABLE III.
Serum Concentrations of Aureomycin and Chloramphenicol in the Same 6 Subjects After a Single Oral Dose of 50 mg/kg.

Time after administration		Serum conc. in μg/cc Aureomycin	Chloramphenicol
15 min.	Sera	3	3
	Range	<1.5	<12.5–13.2
	Median	<1.5	<12.5
30 "	Sera	3	6
	Range	<1.5	<12.5–26.6
	Median	<1.5	18.3
1 hr	Sera	6	6
	Range	<1.5–2	<12.5–33
	Median	1.5	25.5
2 "	Sera	6	6
	Range	2–4	<12.5–40
	Median	3.3	33
4 "	Sera	3	3
	Range	3.3–8.3	33–50
	Median	6.2	50
6 "	Sera	3	3
	Range	2.5–12.5	25–33
	Median	5	25
8 "	Sera	6	6
	Range	2–6.2	16–26.6
	Median	3.2	22.5
24 "	Sera	3	3
	Range	<1.5	<12.5
	Median	<1.5	<12.5

N.B. 1.5 μg/cc = minimal measurable concentration of aureomycin.
12.5 μg/cc = minimal measurable concentration of chloramphenicol.

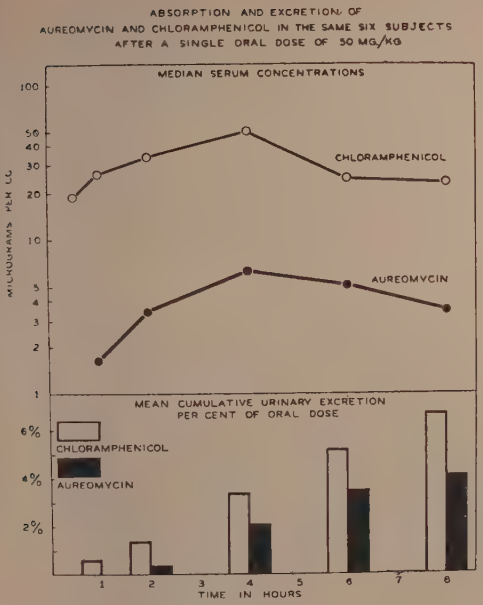

FIG. 2.

therapy. Twenty-two determinations of terramycin in the serum were made in 13 patients who were receiving terramycin hydrochloride in total daily doses of 5 g by mouth given in divided amounts at 6 hour intervals. On a weight basis this represented a daily intake of drug of between 50 and 100 mg per kilo. Most of these patients were suffering from a variety of infections and were febrile at the start of therapy. Measurable amounts of drug were quickly attained in the serum with these doses and ranged from 2.5 to 12.6 μg per ml. With maintenance doses of 4 g per day, the drug could frequently not be measured in the serum. The assay method was not capable, however, of accurately measuring concentrations of terramycin in the serum which were below 2.5 μg per ml. Thirty-five serum determinations of aureomycin were made in 12 patients receiving daily doses of 3 to 12 g of aureomycin hydrochloride given orally in divided amounts. These doses were equivalent to 50 to 200 mg per kilo per day. Several of these patients, especially those receiving the larger doses, were vomiting, however, and did not always retain the whole amount of drug given each day. Serum concentrations of 2.1 to 12.8 μg per ml were attained in patients receiving 200 mg per kilo per day, 3.2 to 10.2 μg per ml in those receiving 100 mg per kilo per day, and 1.5 to 5.1 μg per ml in those receiving 50 mg per kilo per day. Ninety-three determinations of chloramphenicol were made in the sera from 18 patients. The treatment regimens were the same as in the patients receiving maintenance doses of aureomycin. Observations were made on 4 dosage schedules. Maximum concentrations of 50 to 100 μg per ml were found in patients receiving 100 or 200 mg per kilo per day. With doses of 50 mg per kilo per day, amounts of drug in the sera ranged from 6.2 to 100 μg per ml, but most sera contained fewer than 25 μg per ml; with doses of 25 mg per kilo per day the concentration of drug was usually too low to measure, although a few patients attained serum concentrations as high as 12.5 μg per ml.

Concentrations in other body fluids. Small amounts of terramycin were found to be present in the cerebrospinal fluids of 4 patients after oral doses of terramycin hydrochloride. Two of these patients had been receiving maintenance doses of 4 g per day for several days before lumbar puncture. Although no drug was detectable in the sera at the time of lumbar puncture, concentrations of 0.5 and 0.7 μg per ml respectively were found in the cerebrospinal fluid. In 2 patients who had received only 3 g and 750 mg orally for a single day preceding lumbar puncture respectively, no drug could be measured in the cerebrospinal fluid. Three other patients received a single oral dose of 2.5 g 4 hours preceding lumbar puncture. In 2 of these terramycin was present in the cerebrospinal fluid in concentrations of 0.6 and 0.7 μg per ml and simultaneously in the serum in concentrations of 9.5 and 2.5 μg per ml respectively. In the third patient no drug was measured in the cerebrospinal fluid in spite of a serum concentration of 5 μg per ml. The assay procedure was capable of measuring as little as 0.5 μg per ml in the cerebrospinal fluid in contrast to not less than 2.5 μg per ml in the serum. Pleural fluid for terramycin assay was obtained from a patient 2 and 7 hours

TABLE IV.
Urinary Excretion of Aureomycin and Chloramphenicol in the Same 6 Subjects After a Single Oral Dose of 50 mg/kg.

Grouped urine, hr		Urine conc. in μg/cc		Hr after administ.	% of dose recovered in urine	
		Aureomycin	Chloramphenicol		Aureomycin	Chloramphenicol
0–½	No.	6	6	½	6	6
	Range	.8–.15	10–133		.0–.1	.0–.2
	Median	3.8	48		.0	.0
½–1	No.	6	6	1	6	6
	Range	16.5–63	13.3–1250		.0–.2	.0–1.1
	Median	48	175		.0	0.6
1–2	No.	6	6	2	6	6
	Range	50–380	20–1250		.2–.7	1–2.2
	Median	112	275		.3	1.2
2–4	No.	3	3	4	3	3
	Range	284–760	375–1875		1.6–2.4	2.9–3.7
	Median	568	625		2.4	3.7
4–6	No.	3	3	6	3	3
	Range	350–950	825–1875		2.3–4.4	4.7–5.6
	Median	570	1250		3.8	5.6
6–8	No.	3	3	8	6	6
	Range	175–1520	625–1250		1.7–8.4	3.9–9.3
	Median	570	825		3.1	7
23–24	No.	3	3			
	Range	33–100	<10–67			
	Median	33	20			
47–48	No.	3	3			
	Range	6.6–24	<10			
	Median	20	<10			

respectively following the ingestion of a single dose of 1.25 g of terramycin hydrochloride. Terramycin was present in concentration of 512 μg per ml in the 2 hour specimen but could not be detected in the 7 hour specimen. A concentration of 2.5 μg per ml of terramycin was obtained in the ascitic fluid of a patient who had been receiving 3 g by mouth daily for 7 days prior to paracentesis.

Aureomycin could not be measured in the cerebrospinal fluids of 4 patients who had been receiving daily oral doses of 4 g for several days prior to lumbar puncture. An additional patient was given a single dose of 3 g by mouth. Four hours later blood and cerebrospinal fluid specimens were obtained simultaneously for assay. Although the serum concentration of aureomycin was 7.6 μg per ml, no drug was measured in the cerebrospinal fluid. The assay technic was capable of detecting as little as 0.4 μg per ml of aureomycin in the cerebrospinal fluid. One patient with hydrarthrosis of the knee received 4.5 g of aureomycin hydrochloride daily for 3 days. At the end of this time there was present in the joint fluid a concentration of 0.3 μg per ml of the drug. Other observers have reported the accumulation of aureomycin in the cerebrospinal fluid and in other fluids of the body in patients taking the drug by mouth(6).

Nineteen determinations of chloramphenicol were made on cerebrospinal fluids obtained from 7 patients who were receiving daily doses of 25 to 50 mg per kilo of this drug. These patients had concentrations of chloramphenicol in the serum which ranged from less than 6.2 μg to 100 μg per ml. The cerebrospinal fluid concentrations ranged from less than 4 μg per ml, which was the smallest amount detectable by the assay technic, to

6. Dowling, H. F., Lepper, M. H., Caldwell, E. R., Jr., Whelton, R. L., and Brickhouse, R. L., *J. Clin. Invest.*, 1949, v28, 983.

50 μg per ml. All patients receiving the larger doses of chloramphenicol had measurable concentrations of drug in the cerebrospinal fluid after 2 or 3 days of treatment, and these were of the order of one-fourth to one times the serum concentrations. Chloramphenicol was also found in concentration of 12.5 μg per ml in the joint fluid of one patient who had a coexisting serum concentration of 25 μg per ml.

Discussion. It is well recognized that studies of the concentrations of an antimicrobial drug which are attained in the blood may bear only a very loose relationship to the therapeutic results which will be achieved with the administration of that drug. Nevertheless, the results of such studies can be valuable in serving as a guide to the dosage regimens to be employed in therapy and in comparing the absorption and excretion of various forms of the same drug. Of the 3 compounds studied, chloramphenicol provided the most rapid accumulation of antimicrobial substance in the serum and yielded the highest serum concentrations. It also accumulated in the cerebrospinal fluid to the greatest extent. Terramycin was found to compare favorably with chloramphenicol and aureomycin in its absorption following oral administration and in its excretion in the urine. Concentrations of terramycin in the serum were obtained following single large doses or with daily maintenance doses of terramycin hydrochloride which were similar in magnitude to those concentrations attained with equivalent doses of aureomycin given by mouth. The persistence of measurable serum concentrations of terramycin for periods of 6 hours and longer after single doses and the high serum concentrations attained when 1 to 1.25 g maintenance doses of drug were given at 6 hour intervals indicate that total daily doses of 4 to 5 g by mouth will provide satisfactory drug concentrations in the body in terms of the *in vitro* sensitivities of the various microorganisms against which terramycin has been demonstrated to be effective(2).

Summary. A study has been made of the absorption, distribution, and urinary excretion of terramycin, aureomycin, and chloramphenicol in humans. The determinations of absorption and excretion of aureomycin and chloramphenicol following a single oral dose of drug were made in the same subjects. All 3 drugs were readily absorbed after oral administration. The maximum serum concentrations attained after oral doses of 50 mg per kilo were 25 to 50 μg per ml for chloramphenicol, 12 to 16 μg per ml for terramycin, and 3.3 to 12.5 μg per ml for aureomycin. Reasonably high serum concentrations of all 3 drugs were maintained with daily doses of 50 to 100 mg per kilo by mouth. Measurable concentrations of the compounds were obtained in the cerebrospinal fluid and in other body fluids when sufficiently large doses of the drugs had been administered. Urinary excretion of the 3 substances in biologically active form was similar, and high concentrations of drug were attained in the urine.

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Dispersion Oxygenation for Effecting Survival of Dogs Breathing Pure Nitrogen for Prolonged Periods.*† (17873)

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(Introduced by M. B. Visscher)

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It has been a long endeavor of physiologists and clinicians to find satisfactory methods for temporarily replacing a failing circulation or respiration by artificial means outside the body. All previous efforts have been attempts to simulate the actual conditions encountered in the lung, namely spreading venous blood in thin layers over very large surfaces and exposing the blood to oxygen (1-13). With a new type of blood oxygenator, using the principle of gas dispersion for creation of the large surface contact(14), it has been possible to oxygenate blood at a rate sufficient to maintain life in dogs during acute periods of nitrogen breathing extending to 99 minutes. The present report consists of an extension of these experiments in which complete recovery of the animal is used as the criterion of successful oxygenation. In all cases, blood was pumped from the inferior *vena cava* of the animal, to the oxygenator, and back through the jugular vein to the superior *vena cava* of the animal. It was thought that this simple procedure, free from extensive surgical manipulation, and under the conditions of nitrogen inhalation, would offer a clear-cut test of the efficiency of the apparatus. By using survival of the animal as the criterion of the successful experiment, one could determine whether there were any signs of permanent damage to the brain, or other vital organs, as a result of these unusual experimental conditions.

While the apparatus has been modified only slightly since the previous experiments (14), it seems advisable to elaborate somewhat on the principle of the oxygenator itself. Fig. 1, which is largely self-explanatory, is a schematic representation of the conditions in the instrument. It should be pointed out that "foam", as usually defined (15), is not produced. The term "defoaming" is loosely used merely to indicate that excess

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† The authors are indebted to Mr. and Mrs. S. Sergius Vernet, Vernay Patents Co., for financial aid; to Dr. Shailer Bass, Dow Corning Co., for samples of various polymethylsiloxane "antifoam compounds"; to Dr. Joseph Seifter, Wyeth Institute, for generous supplies of Paritol; to Dr. W. R. Kirtley, Lilly Research Laboratories for Protamine; to Mr. Fred Hooven for providing the photocell equipment; and to Dr. L. W. Sontag, Director of the Fels Institute, for encouraging these researches.

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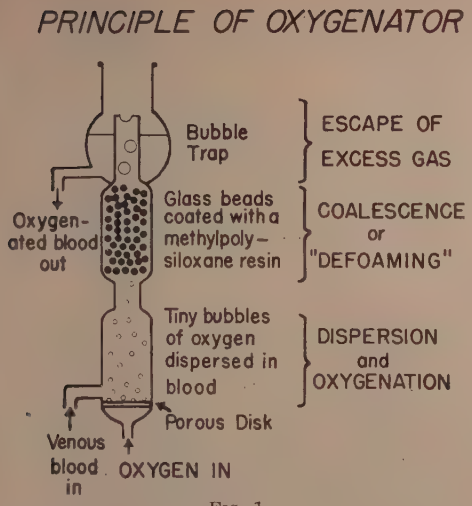

FIG. 1.

gas is removed from the liquid phase. The glass beads should be thoroughly cleaned after about 3 hours use and recoated by stirring the beads with a generous amount of the antifoaming agent. The apparatus was easily sterilized by pumping Dakin's solution and rinsing with sterile saline. Morphine-atropine sedation was used with 1% procaine as a local anesthetic. Paritol (30 mg/kg) was used as an anticoagulant; protamine (about 2 mg/kg) was used to counteract the anticoagulant. Nitrogen was administered from a spirometer and flutter valve system, as previously, through a funnel equipped with a tightly fitting dental dam cuff that extended over the shaved neck of the dog. Arterial pressure, respiratory rate, and electro-cardiac potentials were recorded during the experiments. Blood flow was measured with a rotameter‡, pH with a Beckman Model G meter, and oxygenation of the blood checked by a photoelectric oximeter or Van Slyke determinations(16) as needed. A very satisfactory pump, having a capacity of about 700 ml per minute, and constructed almost

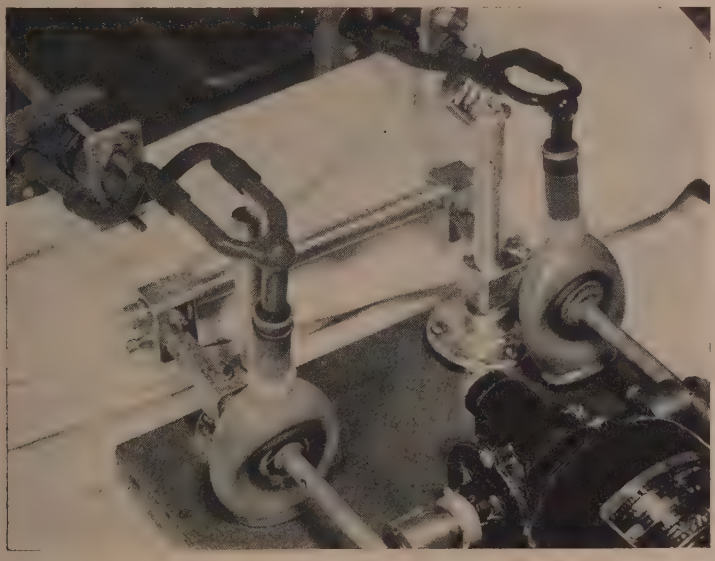

FIG. 2.
Pump constructed of plastic bottles.

‡ The rotameter was not calibrated for each experiment. Therefore, the flow rates given may be assumed to be accurate to only about ±15%. The rotameter provided an extremely valuable and convenient method of making necessary adjustments to obtain maximal or uniform flow during a given experiment, as well as providing absolute flow rates within this limit of error. There still appears to be a disagreement(17,18) as to the method of calibration of rotameters.

TABLE I.
Summary of Experimental Data.

Wt, kg	Mean blood flow, ml/min.	Mean blood flow, per kg ml/min./kg	Duration of breathing N_2 min.	Survival	Cause of death
14.4	600	42	26	+	—
9.1	350	38	60	2 days	Pneumonia
7.0	550	79	25	+	—
7.0	325	46	10	+	—
16.3	300	18	43	10 hr	Pulm. edema
15.8	275	17	61	12 hr	" "
7.7	400	52	60	+	—

entirely of glass and polyethylene, shown in Fig. 2, was used to circulate the blood. It consists of 2 elliptical polyethylene bottles, which were alternately compressed and extended by 2 rods coupled to an eccentric mounted on the shaft of a gear reduction motor (Robbins and Meyers, Springfield, Ohio, 5000 r.p.m., 15-1 gear reduction, 1/20 HP) controlled with a variable transformer. Such a pump is capable of producing negative as well as positive pressures and is rugged enough to function for extended periods of pumping. The pump shown has operated for a total of over 400 hours, using the original set of plastic bottles. Adequate quantities of venous blood can be withdrawn from the inferior *vena cava* by inserting a polyethylene catheter through a femoral vein and placing the tip of the catheter close to the great cardiac vein. This can be accomplished without fluoroscopic guidance by measuring the approximate distance from the femoral incision to the location of the right auricle. The catheter has numerous longitudinal slits for a distance of 6 to 8 cm from the tip to permit the withdrawal of most of the blood flowing along its length. By starting the circulation and oxygenation in the extracorporeal circuit about 5 minutes before the inhalation of pure nitrogen, the respiratory and circulatory signs of acute anoxic anoxia were prevented. When a satisfactory blood flow is maintained, the animals remain conscious throughout the period of nitrogen inhalation, lift their heads if stimulated and retain ocular and bulbar reflexes. The electro-cardiograms which were taken before and during nitrogen inhalation for periods as long as 90 minutes failed to show any signs of general cardiac anoxia.

The present series of experiments (Table I) lends itself to an evaluation of the *minimum* blood flow necessary for the survival of dogs, weighing between 7 and 16.3 kg, and breathing pure nitrogen for periods ranging from 10 to 61 minutes. The 2 large animals, Nos. 74 and 75, received a flow of only about 20 ml of oxygenated blood per kg weight per minute, and died 10 to 12 hours after the experiments, in deep coma. Dog No. 52, weighing 9.1 kg and receiving a blood flow of about 40 ml/kg/min seems to be on the border line since the animal stood up, walked around and reacted to visual and auditory stimuli after the experiment, but later gradually became comatous and finally died of pulmonary edema.

The remaining 4 animals remained conscious during periods of nitrogen inhalation ranging from 10 to 60 minutes, and did not show any respiratory or circulatory signs of anoxia. Their blood flow was always above 40 ml/kg/min. The highest flow in dog No. 59 was about 80 ml/kg/min. The largest surviving animal, who inhaled pure nitrogen for 26 minutes, had a weight of 14.4 kg. Except for the drowsiness due to morphine, the animals behaved in a normal way after being lifted from the operating table. They voluntarily responded to call, and failed to show any clinical signs of cerebral anoxia in the

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following 2 or 3 weeks of observation.

Summary. A new method of blood oxygenation by gas dispersion was tested by subjecting dogs, under local anesthesia and morphine sedation, to conditions of complete anoxic anoxia by substituting pure nitrogen for air as the respiratory gas for about one hour. If a *minimum* blood flow of approximately 40 ml/kg/min was maintained, the animals survived the experiment without loss of consciousness, and recovered completely without exhibiting any neurological signs of anoxic damage.

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A Method with Perfused Feline Hearts of Quantitatively Comparing Drugs with Coronary Vasodilating Activity. (17874)

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Hearts perfused by the method of Langendorff(1) have long been employed for detecting coronary vasodilating action in new compounds. This type of assay would appear to suffer particularly from two limitations: 1. The coronary dilating effects are brief and are difficult to quantitate, so that careful comparisons between congeners are impossible; 2. In many experiments the coronary flow initially is so rapid as to make any further augmentation difficult of attaining. To be assured one is obtaining a primary coronary dilating action, increases in flow must occur without changes in the cardiac rate or the amplitude of the myocardial contractions.

Method. It was found that the proper concentration of a suitable vasoconstrictor in the fluid perfusing the heart would significantly diminish the coronary flow, sometimes by as much as 50%, without altering the rate or the amplitude of the contractions. Originally for this purpose we employed surgical Pituitrin in a concentration varying from .005 to .075 unit per ml. We found it unsatisfactory for 2 reasons: 1. Many hearts proved entirely insensitive to its constrictive effect; 2. Not infrequently its predominant action was a positive inotropic effect with little change in the coronary flow. 2-naphthyl-(1′)-methyl-imidazoline hydrochloride (Privine) yielded better results. Its addition to the recycled perfusion fluid in a concentration of from 1 to 2 γ/ml usually caused in hearts exhibiting a fast initial flow (greater than 15 ml/min) a progressive reduction in the flow up to as much as 50% without altering the rate or amplitude of the contractions. In hearts with slow initial flow (from 10-15 ml/min) relatively high concentrations of Privine (up to 5 γ/ml) usually failed to reduce it further. This proved no disadvantage since such hearts responded directly to coronary dilators. Fig. 1 and 2 illustrate this action by histamine and sodium nitrite respectively. Obviously it is needless to study by this procedure any drugs that do not exhibit a brief augmentation of the coronary flow after single injections of suitable doses directly into the cannula just above the aorta.

Discussion. The higher doses of Privine not infrequently exerted a mild, positive inotropic effect, later followed by irregularities. The last were most common with "irritable" hearts that had exhibited from the beginning a relatively high rate with occasional extra systoles. The coronary constrictive action of Privine would appear weak not only on the basis of the data presented but also on the basis of Meier's work with cats *in vivo* (2). He found Privine invariably augmented the coronary flow, presumably because the elevated blood pressure following its admin-

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2. Meier, R., personal communication.

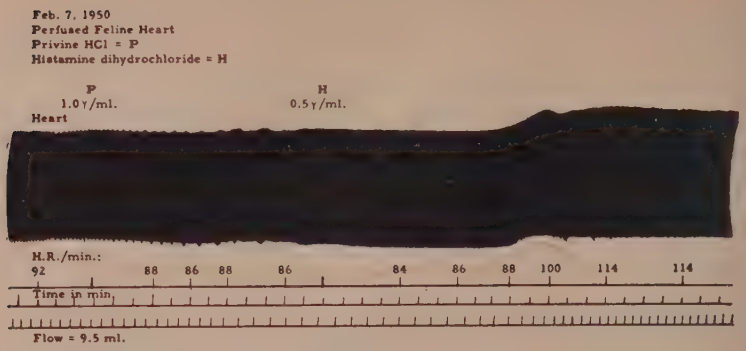

FIG. 1.

Each mark on the flow line indicates passage of a volume of 9.5 ml through the coronary circulation. The rapid initial flow was diminished by addition of 1 γ/ml of Privine to the perfusion fluid. The addition of 0.5 γ/ml of histamine dihydrochloride to the perfusion fluid not only restored the coronary flow but elevated it even above the control rate. The moderate tachycardia it induced could not alone account for the marked change in flow.

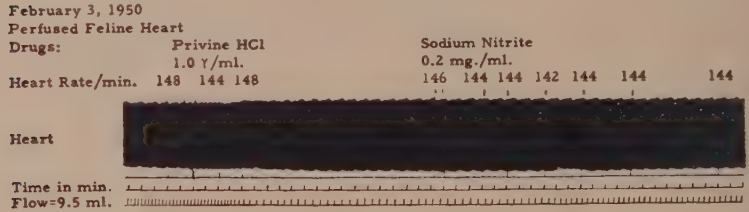

FIG. 2.

Each mark on the flow line indicates passage of 9.5 ml through the coronary circulation. The rapid initial flow was diminished by addition of 1 γ/ml of Privine to the perfusion fluid. The effect of the latter drug was subsequently antagonized by the addition to the perfusion fluid of 200 γ/ml of sodium nitrite.

istration was more than sufficient to offset its slight coronary constrictive action. It would be desirable to know the mechanism of Privine's action, as that would permit a better evaluation of the dilator action of drugs that antagonize it, but for the present one can only state that known coronary dilators do antagonize it. Since it produces constriction more consistently than Pituitrin, it would appear to act closer to the final link in the enzymatic chain of reactions terminating in the contractile response of the vascular musculature.

The method herein presented would appear best suited for a study of the action of a drug upon "tone" of the coronary vessels. Whether a drug effecting an increased coronary flow by this mechanism would be suited for clinical trial must, of course, depend upon its toxicity and general systemic effects. The possible value of this method of selecting a coronary dilator can only be settled after an extensive clinical trial of drugs causing dilatation under the conditions described.

Summary. The use of Privine has been described for inhibiting the coronary flow of feline hearts perfused by the method of Langendorff. This permits a quantitative evaluation of the activity of coronary vasodilators in that majority of perfused hearts that exhibit such a rapid spontaneous coronary flow that its augmentation is difficult, if not impossible. Under the conditions described the Privine does not alter the cardiac rate or the amplitude of the myocardial excursions.

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Affinity of Avidin for Biocytin. (17875)

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The isolation of a crystalline complex of biotin, termed biocytin, from yeast extract recently has been announced(1). Pursuant to studies on the metabolic significance of biocytin the affinity of avidin for biocytin has been investigated. In these studies a quantitative method(2,3), whereby the affinity of avidin for biocytin is compared with the affinity of the protein for biotin, was employed. The procedures used involved the addition of avidin to mixtures of biotin and biocytin. The biotin and avidin were used in approximately stoichiometric amounts while the amount of biocytin was varied. Under these conditions the amount of biotin available for the growth of biotin-requiring species is equivalent to the amount of biocytin taken up by the avidin. The relative affinity then is expressed arbitrarily as the ratio of the concentration of biocytin to biotin at which one-half of the biotin remains free and available for the growth of the test organism. The ratio will be low for a compound for which avidin has considerable affinity and high for a compound that does not combine readily with avidin. For these studies a biotin-requiring microorganism that is incapable of responding to biocytin as such must be employed.

Experimental. The methods employed in this study were those commonly used in microbiological assays with lactic acid bacteria. *Lactobacillus arabinosus* was used as the assay organism in conjunction with a previously described medium(4). The extent of bacterial growth was determined turbidimetrically after 24-36 hours of incubation at 30°C.

Three different sources of avidin were employed in this study, (a) sterile egg white, (b) a commercial concentrate containing 50 units of activity per gram (SMACO), and (c) a highly purified preparation (kindly supplied by Dr. Fraenkel-Conrat of the Western Regional Research Laboratory.) Sterilization of avidin solutions by filtration through fritted glass filters resulted in unpredictable adsorption of activity. Finally it was found satisfactory to weigh or to pipette out each avidin preparation into sterile water and to use with aseptic technic aliquots of each solution.

Combinability studies were carried out in the microbiological assay medium after autoclaving and just prior to seeding by the addition of avidin to tubes containing biotin or a combination of biotin and biocytin (Table I).

Separate studies have shown that the sample of crystalline biocytin(1) used in these studies had no demonstrable biotin activity either alone (up to 0.5 γ/tube) or in combination with an amount of biotin (0.001 γ/tube) promoting one-half maximal growth of *Lactobacillus arabinosus*.

Results and discussion. Typical data required for the calculation of the affinity ratio of avidin for biocytin are summarized in Table I. These data were obtained using the commercial concentrate as the avidin source.

It is not always possible to use in the combinability phase of an experiment (tubes 12-19) an amount of avidin that combines exactly with the level of biotin employed. Indeed for all practical purposes it is not essential that the two be used in exactly stoichiometric amounts. In this experiment biotin was in slight excess. Prior to further consideration of the data the biotin added in slight excess, that is the biotin with which

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TABLE I.
Protocol for Avidin Combinability Studies.

Tube No.	Biotin, γ/tube	Biocytin, γ/tube	Avidin, γ/tube	Turbidity
1	0			52
2	.00025			115
3	.00050			170
4	.00075			204
5	.00100			248
6	.00150			294
7	.00250			370
8	.0025		10	330
9	.0025		25	250
10	.0025		50	70
11	.0025		100	67
12	.0025	.0025	50	197
13	.0025	.0050	50	254
14	.0025	.0075	50	272
15	.0025	.0125	50	300
16	.0025	.025	50	325
17	.0025	.050	50	345
18	.0025	.075	50	360
19	.0025	.125	50	360

50γ of avidin concentrate did not quite combine (tube 10) is subtracted from that found present in the various mixtures of avidin, biotin and biocytin (tubes 12-19). After subtraction of the biotin in excess of the avidin equivalence, the percentage of added biotin available for growth of *Lactobacillus arabinosus* (equivalent to the biocytin combined in the avidin) is plotted as a function of added biocytin. A curve illustrated by Fig. 1 thus is obtained. By definition the affinity ratio is the ratio of the concentration of biocytin to biotin at which one-half (50%) of the biotin remains free and available for the growth of the organism. Thus in this experiment the level of biocytin required to attain this condition is approximately 0.0070γ/tube (abscissa scale). The total concentration of biotin is approximately 0.00245γ/tube (ordinate scale) after correction for the biotin used in excess of that combining with 50γ of the avidin concentrate employed. The affinity ratio in this experiment is about 3. The affinity ratios obtained were independent of the avidin source used. In 5 experiments employing avidin from 3 sources described an average affinity ratio of 4.1, range 2.9-5.0, was obtained. Thus avidin has a greater affinity for biotin than for biocytin.

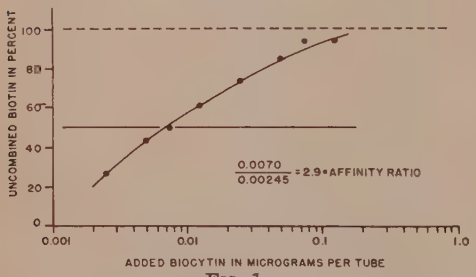

FIG. 1.
The relative affinity of avidin for biotin and biocytin.

Summary. Avidin has a greater affinity for biotin than for biocytin. The affinity ratio for biocytin, as previously defined, is about 4.

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Apparent Free Histidine Plasma and Urine Values in Rheumatoid Arthritis Treated with Cortisone and ACTH.* (17876)

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Preliminary observations(1,2,3,4) on the urinary excretion and plasma levels of amino acids in rheumatoid arthritis made it seem logical to determine these values before, during and after treatment with the new antirheumatic agents described by Hench *et al.* (5).

This paper summarizes a study of 15 patients with active rheumatoid arthritis treated with 17 - hydroxy - 11 - dehydrocorticosterone (Cortisone)† and adrenocorticotrophic hormone (ACTH)‡ and reports urinary excretions and plasma levels of histidine during active disease and through remission. This group was uncomplicated by any other medication.§

Experimental. Five patients (2 females and 3 males) were treated with Cortisone. Ten patients (8 females and 2 males) were treated with ACTH. The average age was 50.8 years with a range of 27 to 70 years. The average duration of the disease was 5.1 years with a range of 1.5 to 16 years. All patients suffered from active rheumatoid arthritis, had been under observation by the investigators for 6 months or longer, and were hospitalized during the entire period of investigation. They were observed during an initial pre-treatment period and were under metabolic control during the entire experimental period. The evaluation of therapy response was both subjective and objective. Stiffness, rest pain, and motion pain were determined by the patients' answers. Heat, swelling, redness, tenderness, strength of grip and degrees of function were determined daily by inspection and measurement. Blood pressure and weight recordings were made daily. Complete blood counts and sedimentation rates were performed at selected intervals. Cortisone was given in an initial dose of 300 mg intramuscularly and on each successive day 100 mg of the preparation was used. ACTH was given intramuscularly in variable dosage for 8 to 17 days. Daily dosage varied from 40 to 160 mg per day and was given in divided doses every 6 hours. Subsequent cases have been found to experience satis-

* This study was supported in part by a grant from the USPHS and The Fair Foundation.

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4. *Proceedings of the First Clinical ACTH Conference,* John R. Mote, M.D., Editor, The Blakiston Company, Philadelphia, 1950, p. 386.

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† All Cortisone was provided by Merck and Co.

‡ All ACTH was provided by Armour and Co. (Dr. John Mote, Medical Director).

§ During the past year a total of 51 patients with rheumatoid arthritis have been studied, 14 receiving Cortisone and 37 ACTH. In attempting to accelerate and prolong the favorable effects of treatment, a number of this group received during the control period and/or through the treatment period, various agents such as ascorbic acid, testosterone, benedryl, and adenosine triphosphate (ATP), thyroid, adrenalin, gold, X-ray therapy, amino acid feeding and aspirin. Amino acid studies were done on this group of patients but are not being reported at this time. However, we should like to state that the urinary histidine excretion response to be described has not occurred in this group except in association with clinical remission.

TABLE I.
Excretion of Apparent Free Histidine During Cortisone Administration.
(5 patients with rheumatoid arthritis).

Patient	Total dosage, mg	Control values, mg per 24 hr	Treatment values, mg per 24 hr	Diff. between max. (control and treatment) mg per 24 hr
B	1600 (14)*	53 (41– 73)	126 (117–136)	63
Y	1800 (14)*	57 (47– 62)	144 (101–186)	124
S	1000 (8)*	63 (53– 73)	131 (104–148)	75
N	1000 (8)*	98 (95–100)	149 (110–167)	67
R	1000 (8)*	84 (54–114)	141 (64–180)	66
Avg	1280 (10.4)*	71 (58– 86)	138 (99–163)	77

* Days on treatment.

TABLE II.
Excretion of Apparent Free Histidine During ACTH Administration.
(10 patients with rheumatoid arthritis).

Patient	Total dosage, mg	Control values, mg per 24 hr	Treatment values, mg per 24 hr	Diff. between max. (control and treatment) mg per 24 hr
K	310 (15)*	23 (20– 26)	81 (62–117)	91
D	675 (17)*	104 (104–104)	224 (143–334)	230
G	640 (11)*	63 (60– 66)	133 (81–174)	108
L	680 (11)*	70 (64– 76)	201 (124–285)	209
P	600 (11)*	62 (61– 62)	166 (118–231)	169
K	620 (11)*	119 (97–140)	176 (134–260)	120
B	370 (12)*	23 (18– 28)	157 (109–211)	183
W	380 (12)*	53 (44– 61)	144 (96–175)	114
F	370 (12)*	30 (23– 37)	106 (54–146)	109
M	960 (11)*	83 (79– 87)	374 (148–493)	406
Avg	560.5 (12.3)*	63 (57– 69)	176 (107–243)	174

* Days on treatment.

factory remissions on 40 mg (or less) ACTH per day.

Collection of urine and blood specimens. Twenty-four hour urine specimens were collected under toluene, diluted to appropriate volume and aliquots stored at -15°C. Fasting blood was obtained by venipuncture and prevented from clotting with heparin. Tungstic acid filtrates were prepared according to the method of Hier and Bergeim(6,7) and stored at -15°C. A modification of the microbiological technic of Henderson and Snell (8) was used for the assay of free histidine. Hydrous histidine hydrochloride ($C_6H_9N_3O_2$·

6. Hier, S. W., and Bergeim, O., *J. Biol. Chem.*, 1945, v161, 717.

7. Hier, S. W., and Bergeim, O., *J. Biol. Chem.*, 1946, v163, 129.

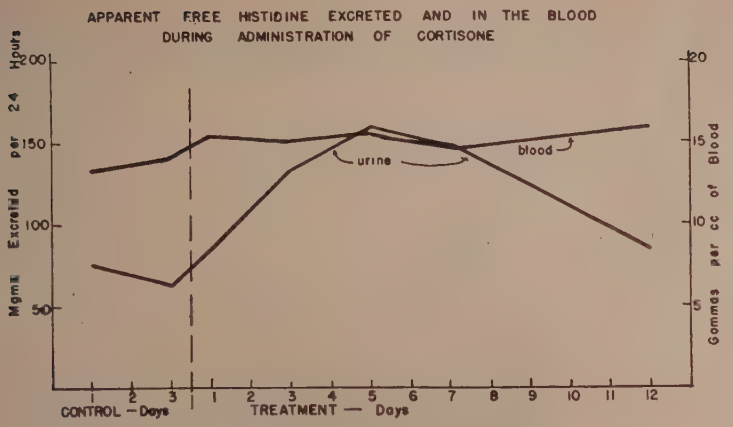

FIG. 1.
Average of 2 ♀ and 3 ♂ rheumatoid arthritics.

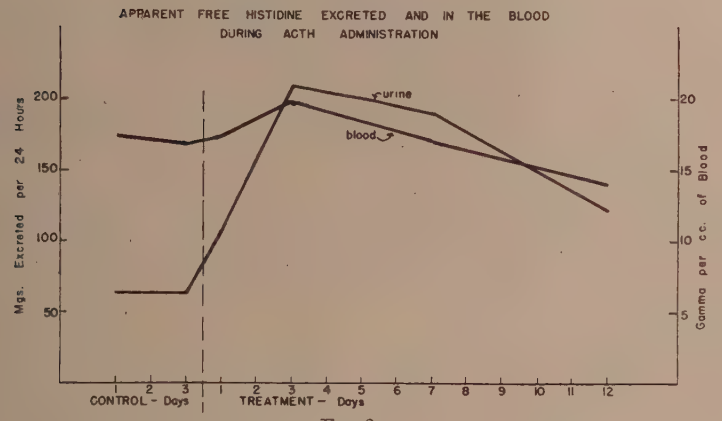

FIG. 2.
Average 8 ♀ and 2 ♂ with rheumatoid arthritis.

$HCL{\cdot}H_2O$) ("Merck") was used as a standard. All values of apparent free histidine are expressed in terms of this compound. The test organism was *Leuconostoc mesenteroides P-60*. All specimens from each patient were assayed in one series to eliminate errors due to variations in organism response. Cortisone and ACTH were found to have no stimulatory or inhibitory effect on the microorganisms.

8. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, 1948, v172, 15.

Clinical results. All of the patients experienced improvement within 24-48 hours following the initial injection of either hormone. The patients described a sense of well-being, decrease in joint stiffness, rest pain, motion pain and joint tenderness. Swelling disappeared slowly over the period of treatment. Strength improved rapidly and there was a marked increase in appetite and energy. Following the initial 48 hour period, improvement was slower but continued steadily as long as the medication was given. The

TABLE III.
Apparent Free Histidine Plasma Values During Cortisone Administration.
(5 patients with rheumatoid arthritis).

Patient	Total dosage, mg	Control values, γ per ml	Treatment values, γ per ml	Diff. between max. (control and treatment) γ per ml
B	1600 (14)*	13.6 (13.2–14.2)	17.5 (13.7–21.3)	7.1
Y	1800 (14)*	12.1 (–12.1)	14.4 (12.1–18.7)	6.6
S	1000 (8)*	16.0 (15.8–16.4)	16.3 (15.6–17.4)	1.0
N	1000 (8)*	14.8 (14.6–14.9)	14.5 (13.0–17.3)	2.4
R	1000 (8)*	13.6 (13.4–13.7)	14.9 (13.7–16.3)	2.6
Avg	1280 (10.4)*	14.0 (13.8–14.3)	15.5 (13.6–18.2)	3.9

* Days on treatment.

sedimentation rate dropped an average of 37.1 mm per hour (Modified Westergren method). No toxic effects were noted except in the one case that received the large dose of 160 mg per day. This patient developed transient hypertension, weight gain and oliguria. In the remaining cases there was no alteration in weight on the constant diet used. Blood pressure did not alter significantly. No edema developed. The pulse rate was not significantly changed. There were no apparent differences in the clinical response to either of the hormones.

Chemical results. Urine. All patients treated with Cortisone or ACTH had a significant rise in apparent free histidine excretion. (Tables I and II) Both control excretion and increase of excretion on medication show considerable individual variation. From the composite graphs (Fig. 1 and 2) it can be seen that apparent free histidine excretion rises upon initiation of medication and continues to rise to a peak. The urinary excretion curves produced by Cortisone and ACTH are comparable except that in the case of the latter, the peak is reached sooner and the increase is of a greater magnitude. While still on either medication, the patients show a decrease from the peak. When medication is discontinued, there is a more rapid fall toward the control levels. This type of curve has occurred only in association with clinical remission. From the data at hand, and within the limits of dosage employed, there is a significant‖ correlation between ACTH dosage and the maximum increase in excretion herein reported(9).

Blood. The average values for histidine in the blood during treatment (Tables III and IV) are higher than the control in 9 patients and lower in 6. However, the maximum values reached during the treatment period are significantly higher‖ than the highest control values(10). Fig. 1 and 2 present the average plasma levels before, during and after treatment with Cortisone and ACTH. While the differences in plasma levels reported are small it seems logical to assume that even a small rise in plasma levels would be commensurate with high urinary excretion values. The plasma levels are representative of values existing during only one period of the day while the excretion values provide a measure of the total 24-hour urinary excretion. Furthermore, expressed in terms of average blood volumes a rise of 5γ per ml would represent a total increase of approximately 25 mg. The possible relationship of these findings to changes in kidney function or to other changes in metabolism produced by ACTH or Cortisone have not

‖ $P < .05$ considered significant.

9. Wallace, H. A., and Snedecor, G. W., *Iowa State College Bulletin*, 1931, v30, 8.

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TABLE IV.
Apparent Free Histidine Plasma Values During ACTH Administration.
(10 patients with rheumatoid arthritis).

Patient	Total dosage, mg	Control values, γ per ml	Treatment values, γ per ml	Diff. between max. (control and treatment) γ per ml
H	310 (15)*	15.5 (15.3–15.8)	16.4 (11.5–18.1)	2.3
D	675 (17)*	16.4 (15.8–17.4)	16.2 (15.1–17.6)	0.2
G	640 (11)*	17.2 (15.7–18.7)	22.1 (19.4–23.9)	5.2
L	680 (11)*	19.0 (18.3–19.7)	18.5 (16.9–19.9)	0.2
P	600 (11)*	15.1 (15.0–15.2)	13.3 (12.3–14.5)	−0.7
K	620 (11)*	16.9 (16.7–17.0)	18.9 (18.3–19.9)	2.9
B	370 (12)*	19.7 (19.3–20.1)	16.3 (13.0–18.3)	−1.8
W	380 (12)*	12.1 (11.1–13.1)	13.1 (8.7–15.6)	2.5
F	370 (12)*	14.5 (14.3–14.7)	12.7 (10.3–14.9)	0.2
F	960 (11)*	24.5 (23.9–25.0)	27.4 (20.0–38.5)	13.5
Avg	560.5 (12.3)	17.1 (16.5–17.7)	17.5 (14.6–20.1)	2.5

* Days on treatment.

as yet been studied adequately.

Summary. 1. Five rheumatoid arthritics treated with Cortisone and 10 treated with ACTH show a striking increase in urinary excretion of apparent free histidine. This increase in histidine urinary excretion has to date occurred only in association with clinical remission.

2. Plasma levels were not significantly altered during the *average* treatment period, but the maximum value reached during treatment showed a significant increase when compared with the highest control values.

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Mucolytic Enzyme Systems XIII. Effect of Compounds on Hyaluronidase and its Inhibition by Human Serum.* (17877)

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During the course of the investigations on hyaluronidase and its inhibition, previously reported, further modifications of the procedures for the preparation of the substrate and the viscosimetric measurement of the hyaluronidase activity were instituted. These, as well as the effects of certain ions and or-

* This investigation was supported by a research grant from the National Institutes of Health, Public Health Service, and the Medical Research Fund of the Graduate School, University of Minnesota.

ganic compounds on both the testicular enzyme activity and its non-antibody inhibitor in human blood serum, are now being reported. It has been established that salts exert a definite influence on hyaluronidase activity(1-7), and it is clear that the kind and degree of the influence depends largely on the salt concentration, the source and degree of purification of the enzyme and the substrate, and the method of determining the activity. These factors also affect the activity of the hyaluronidase inhibitor in blood serum(8-11). Haas(8) found that phosphate inhibits the hyaluronidase inhibiting property of serum, and Baumberger and Fried (9) showed that magnesium ions potentiate the serum inhibitor. Subsequently, the magnesium effect was studied more fully by Freeman *et al.*(11) who employed a turbidimetric method. In the present studies the viscosimetric method was used to observe the influence of phosphate and magnesium ions, different buffers, sulfhydryl radicals, cyanide, azide, histamine, benedryl, toluidine blue, and adrenocorticotrophic hormone (ACTH).

Methods. During warm weather, particularly, it had been found that batches of substrate solution suffered a spontaneous decrease in viscosity even though the solutions were stored in a refrigerator. The difficulty was traced to contamination with organisms that elaborate hyaluronidase. Accordingly, the procedure for making up the substrate solution was altered as follows: Approximately 2.5-3.0 g of hyaluronic acid powder prepared from human umbilical cords(12) was added to 250 ml of a buffer solution (6 vol. 0.1 M veronal buffer, pH 6.7, + 1 vol. 2.0 M NaCl) in a Waring blendor. After 1-2 min. agitation the mixture was poured out, 250 ml of buffer was used to rinse the blending vessel, and the rinsing liquid was added to the substrate solution in a flask which was then stoppered. This flask was heated at 65° for 30 min., allowed to stand at room temperature overnight, again heated at 65° for 30 min., and, while hot, passed first through a coarse porosity sintered glass filter, then through one of medium porosity. After allowing the filtrate to stand overnight, it is heated once more at 65° for 30 min. The substrate solution is now a little more viscous than desired, hence it is carefully diluted with the veronal-saline buffer until the "relative viscosity"(13) becomes 1.5. A veronal buffer is used with the substrate instead of the mixed phosphate-acetate buffers previously employed(13) since the veronal increased the sensitivity of both the enzyme and inhibitor measurements. The enzyme preparation was made as previously described(13) and the activity was adjusted (by either dilution with the buffer or strengthening with more bull testes powder) to give an R_o value of 240 sec. R_o is defined as the time required to reduce the "relative viscosity" of the reaction mixture to half its initial value. The final reaction mixture was prepared by adding 1.5 ml of the aqueous solution of the substance to be tested to 0.5 ml of the buffered enzyme, allowing to stand 6-10 min. at 38°, and then 4.0 ml of the buffered substrate was added. 5.0 ml of the mixture was pipetted into a viscosity tube for the measurements. The results were expressed as percent inhibition or activation of the hyaluronidase(12). For work on the serum inhibitor 0.04 ml of

1. Robertson, W. B., Ropes, M. W., and Bauer, W., *J. Biol. Chem.*, 1940, v133, 261.

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10. Dorfman, A., Ott, M. L., and Whitney, R., *J. Biol. Chem.*, 1948, v174, 621.

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13. Glick, D., and Gollan, F., *J. Inf. Dis.*, 1948, v83, 200.

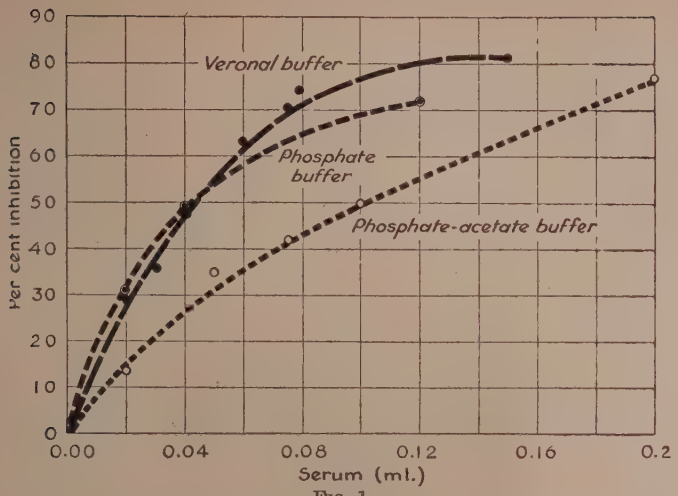

FIG. 1.

pooled normal human serum was diluted to 1.5 ml with distilled water. When the effect of a substance on the serum inhibitor was studied, the substance was first added to the serum solution before the latter was mixed with the enzyme. In those cases where the substance was acidic, the dissolved material was brought to pH 6.7 with 0.2 N NaOH before it was added to the serum solution.

Obtaining serum from a drop or two of blood for work on small animals or infants was carried out with the collaboration of Dr. M. L. Grais, as follows: Blood from a skin puncture was drawn up into glass tubing (10 cm long, 3 mm outer diam., 1.5 mm inner diam.), both ends of the tube were plugged with paraffin at once. After clotting, the tube was centrifuged, cut at the juncture of the packed cells and serum, and a serum aliquot was removed with a constriction pipette (14, p. 172). It was found in a number of cases that a sample obtained in this fashion from a heel prick in an infant showed no significant difference in the inhibitor concentration from a sample obtained by venipuncture of the same individual.

Results and discussion. Effect of buffer. A greater enzyme activity was observed when the phosphate-acetate buffer originally used (13) in the substrate solution (2 vol. 0.5 M phosphate, pH 7.0, 4 vol. 0.02 M acetate, pH 4.7, 1 vol. 2 M NaCl) was replaced by the veronal buffer described in this paper, or by a phosphate buffer (6 vol. 0.1 M phosphate, pH 6.7, 1 vol. 2 M NaCl), Fig. 1. At a pH of 6.7 veronal is at the acid end of its buffering range while phosphate is in the middle of its range. However, checks of the pH before and after the enzymatic hydrolysis of hyaluronic acid in solutions containing either buffer showed no change. Veronal was adopted in preference to the phosphate since the veronal was less favorable to the development of microorganisms.

Effect of magnesium and phosphate ions. The potentiating effect of low concentrations of magnesium ions on the serum inhibitor and the inhibiting effect of higher concentrations is illustrated in Fig. 2. The magnesium ion donated by the serum itself is negligible ($<1.5 \times 10^{-5}$ mEq./ml reaction mixture). Over the concentration range given in Fig. 2 it was found that magnesium ions had no direct influence on the activity of the hyaluronidase. An increasing direct inhibition of the enzyme was observed, however, with increasing concentrations of magnesium ion above 0.01 mEq. per ml reaction mixture. The influence of magnesium ions relative to

14. Glick, D., Techniques of Histo- and Cytochemistry, Interscience Publishers, Inc., New York, 1949.

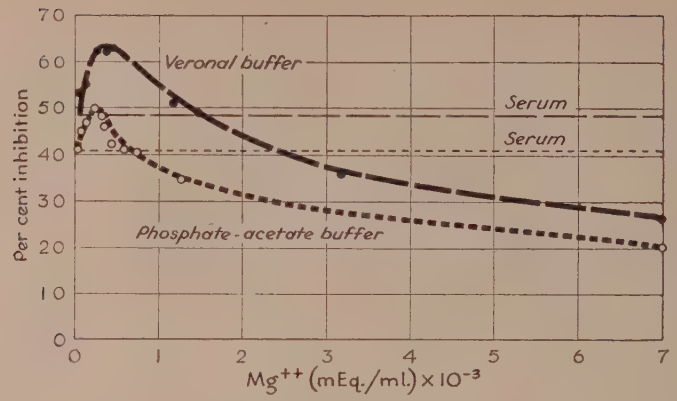

FIG. 2.

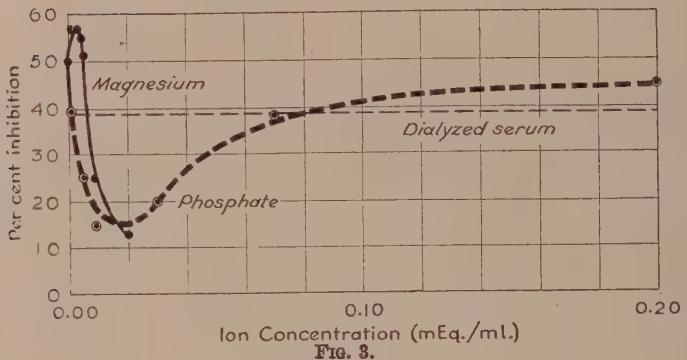

FIG. 3.

phosphate on serum dialyzed for 3 days at 6°, and chloride free, is illustrated in Fig. 3. From Fig. 4 it appears that the action of the serum inhibitor is reduced by a low concentration of phosphate, and then with increasing concentrations the inhibition is due solely to the effect of the ion on the hyaluronidase. The phosphate in the serum used would contribute about 2.5×10^{-5} mEq. per ml reaction mixture, and is therefore negligible.

Effect of sulfhydryl radicals. To determine whether sulfhydryl radicals are necessary for the hyaluronidase action, the effect of compounds that react with these groups was investigated. Chain and Duthie(15) had reported that 0.05 M iodoacetate inhibited the enzyme. It may be seen from Fig. 5 that iodoacetate destroys the serum inhibition while at the same time it inhibits the hyaluronidase. This would seem to implicate sulfhydryl in both the enzyme and inhibitor actions. The effect of other substances that react with sulfhydryl groups is given in Table I, No. 1-3. It is apparent that at the low concentrations used, these compounds have little direct effect on the hyaluronidase, and Nos. 1 and 3 exhibit a reduction of the serum inhibition. Whether No. 2 failed to affect the serum inhibitor because of the low concentration employed is not known. It was observed that in the presence of free cysteine or glutathione, hyaluronic acid spontaneously depolymerized. This is illustrated in Table II, and it serves to point up a disagreement with the results of Robertson *et al.*(16) who indicated that these substances are without

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16. Robertson, W. B., Ropes, M. W., and Bauer, W., *Biochem. J.*, 1941, v35, 903.

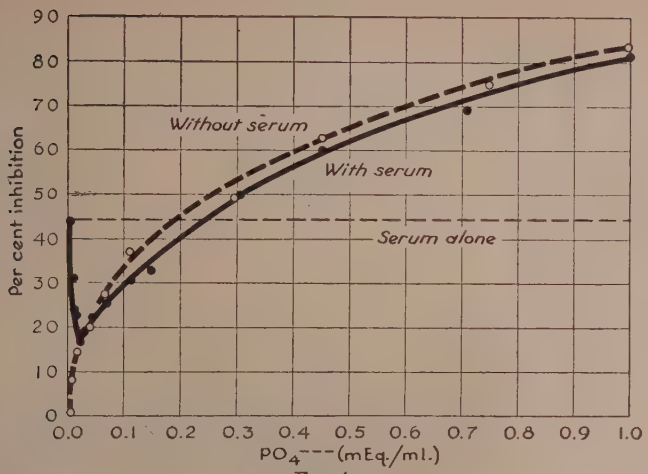

FIG. 4.

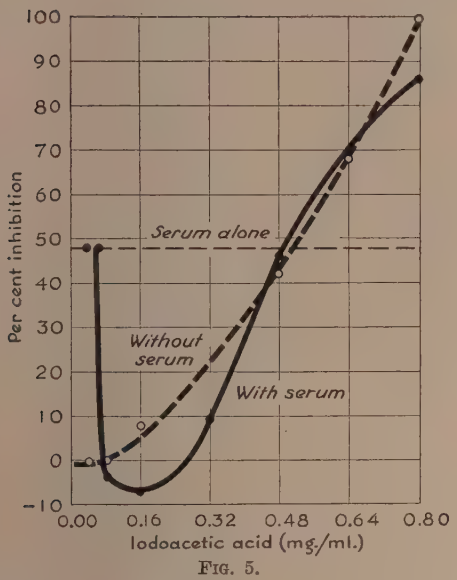

FIG. 5.

the depolymerizing effect; while it is not in disagreement with the work of Skanse and Sundblad(17) who found that the depolymerization by oxygen was enhanced by cysteine in the presence of traces of copper. The action of these reducing agents is similar to that of others(17), ascorbic acid and its oxidation products(3,15,16), copper(18,19), and azoproteins(20,21). Methionine, Table I, did not affect the viscosity of the substrate; at the concentration used, it did inhibit the enzyme and appeared to destroy most of the serum inhibitor.

Effect of other compounds. From Fig. 6 it may be seen that cyanide both destroys the serum inhibitor and inhibits the hyaluronidase. Chain and Duthie(15) had reported 0.05 M cyanide to be without effect on hyaluronidase, and Skanse and Sundblad(17) found that cyanide as well as diethyldithiocarbamate inhibited the depolymerization effected by oxygen, presumably by blocking heavy metal catalysis of this reaction. Sodium azide, Table I, also inhibits the enzyme in addition to inactivating the serum inhibitor, and the same applies to histamine and benedryl. Under the conditions they employed, Moynahan and Watson(22) found very little *in vitro* inhibition by benedryl and other antihistaminics on testicular hyaluronidase. Mayer and Kull(23) reported that, *in vivo*,

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19. Pirie, A., *Brit. J. Exp. Path.*, 1942, v23, 277.

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21. Favilli, G., *Nature*, 1940, v145, 866.

22. Moynahan, E. J., and Watson, D., *Nature*, 1949, v163, 173.

23. Mayer, R. L., and Kull, F. C., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 392.

TABLE I.
Effect of Certain Compounds on the Hyaluronidase Activity and on the Serum Inhibitor.

No.	Compound	Amt per ml reaction mixture	% inhibition: Compound alone	% inhibition: Compound with serum	% inhibition: Serum alone
1	p-chloromercuribenzoic acid†	0.25 ml saturated soln.*	7	37	47
2	o-iodosobenzoic acid†	" " "	7	51	47
3	phenylmercuric nitrate	" " "	14	16	46
4	DL-methionine	12 mg	46	54	48
5	Sodium azide	2 "	31	39	53
6	Histamine dihydrochloride	2.5 "	17	26	46
7	Benedryl	2.5 "	17	17	46

* Saturated at 25°.
† Kindly supplied by Dr. L. Hellerman, Johns Hopkins University.

TABLE II.
Effect of Cysteine and Glutathione on the Viscosity of a Hyaluronic Acid Solution at 38°C.

Time from start of experiment	"Relative viscosity"*: Cysteine (5.1 mg cysteine HCl per ml reaction mixture)	"Relative viscosity"*: Glutathione (5.8 mg per ml reaction mixture)
0	1.50	1.50
5 min.	1.46	1.49
20 "	1.44	1.48
3 hr	1.14	1.33
11 "	0.79	0.94
18 "	0.56	0.69

* $\left(\frac{T_1-T_2}{T_1}\right)$ where T_1 equals initial outflow time from viscosity tube for complete reaction mixture, and T_2 equals outflow time for same mixture without substrate (12).

both pyribenzamine and antistine inhibited the spreading effect of hyaluronidase, while Swyer(24) observed that histamine increased the spreading action. It would appear that the physiological antagonism between histamine and the antihistaminic compounds is not reflected in a difference in their *in vitro* hyaluronidase inhibiting properties, even though their *in vivo* actions on the spreading phenomena may be in accord with this antagonism. Toluidine blue is of interest since it is a basic compound that might be able to compete with other basic factors in their reactions with hyaluronic acid. Fig. 7 demonstrates that toluidine blue can reduce the serum inhibition, and at the same time inactivate the enzyme. The data might be interpreted as indicating that the basic dye binds the acidic inhibitor and thus inactivates it, while the dye also competes with the enzyme for the acidic groups in the substrate and thus produces a direct inhibition of the enzyme action.

Effect of ACTH†. It has been found that ACTH and the adrenal cortex influence the level of the inhibitor in the serum(25). An *in vitro* test of the effect of ACTH on the

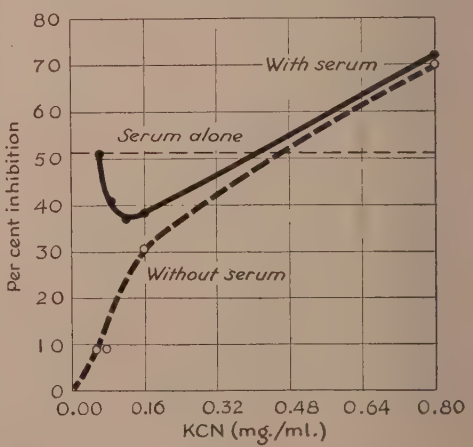

FIG. 6.

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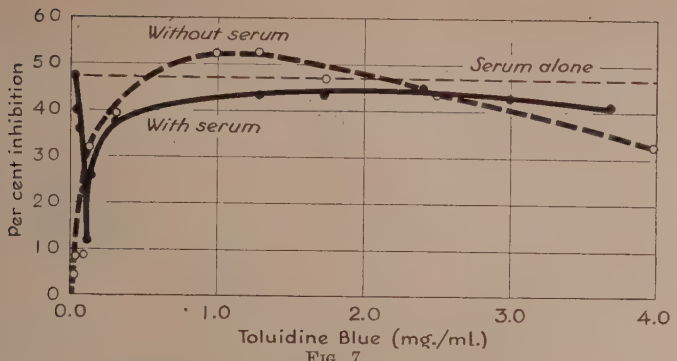

FIG. 7.

inhibitor as well as on the enzyme revealed a negligible influence on either up to the highest concentration used, 2.5 mg per ml reaction mixture. In an earlier investigation(12), cortisone and desoxycorticosterone were also found to be without effect. That adrenal compounds can influence the *in vivo* action of hyaluronidase as a "spreading factor" appears from the work of Opsahl(26) and Seifter *et al.*(27).

Summary. Modifications in the preparation of the substrate for the measurement of hyaluronidase activity by the viscosity method have been described. Increased sensitivity in the measurement of both the enzyme and its inhibitors has resulted, and spontaneous depolymerization of the substrate on storage has been virtually eliminated. The effect of the use of veronal and phosphate buffers in the substrate solution has been investigated, and the influences of a range of concentrations of magnesium and phosphate ions on both hyaluronidase and its serum inhibitor have been determined.

Evidence has been presented to indicate a possible role of sulfhydryl groups in both the enzyme and inhibitor actions. Cysteine and glutathione have been shown to cause a depolymerization of hyaluronic acid.

Methionine, cyanide, azide, histamine, benedryl, and toluidine blue have been found to both inactivate the serum inhibitor and inhibit hyaluronidase. Certain implications have been discussed.

Adrenocorticotrophic hormone has been shown to have negligible effect, *in vitro*, on either hyaluronidase or its serum inhibitor.

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† We are indebted to Drs. John Mote and Edwin E. Hays of the Research Division of Armour and Co., Chicago, for the ACTH used. The material had a potency of 41% of Armour Standard La-I-A and contained 0.002 oxytocic units per mg.

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11-Dehydrocorticosterone Acetate (Compound A) in Normal and Tumor Bearing Mice. (17878)

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It has been shown that 11-dehydro-17-hydroxycorticosterone (Compound E) as well as adrenal cortical extracts have a restraining effect on the growth of leukemias and lymphoid tumors of mice and rats(1,2,3,4). We have found no reference in the literature to such an action of 11-dehydrocorticosterone (Compound A). The study here reported provides evidence that Compound A has pronounced biological effects. Among these is the ability to extend the life of mice bearing a transplantable leukemia and to modify the growth of a lymphosarcoma.

Materials and methods. Normal male and female strain *AK (RIL)* mice when 55 to 69 days of age, young strain *DBA* mice bearing transplantable leukemia P1534, and strain *C3H* mice bearing lymphosarcoma 6C3HED were employed. The 11-dehydrocorticosterone acetate was suspended in saline, 50 mg/cc, and unless otherwise noted, subcutaneous injections of 4 mg/day were used in 2 divided doses. Control mice received similar physiological saline injections. The experiments were restricted by the fact that only a few milligrams of Compound A were available and no more could be obtained. The transplantable leukemia and lymphosarcoma were transferred by subcutaneous injection of the local tumor mass with a 12 gauge trochar. Mice bearing leukemia P1534 typically die suddenly about 10 days after tumor transfer. The local mass of the lymphosarcoma is well enlarged at 6 days and very large at 12 days. Mice bearing this tumor die about 3 weeks after tumor transfer. All mice were maintained at 22°C, fed Purina Fox Checkers *ad libitum* with a supplement of sunflower seeds and rolled oats.

Results. Three criteria have been employed in determining the response of the normal mice to Compound A: (1) body and organ weight, (2) hematologic studies, and (3) histologic studies. The first two are shown in Table I; the latter is reserved for a later report. Although the findings must be considered preliminary, a few points stand out: (1) body weight was well maintained, (2) organs related to the lymphatic system; spleen, thymus, and mesenteric lymph nodes were reduced in size. Females were started in diestrus and autopsied in diestrus. The treated females were acyclic and the non-treated cyclic, and there appears to be some evidence of reduction in accessory reproductive organ weight. Microscopic observation showed the adrenal cortex of males and females to be greatly reduced; in the females the X zone was large and vacuolated.

Four criteria were employed in studying the response of the transplanted leukemia to Compound A: (1) duration of life, (2) hematologic studies, (3) growth of leukemic cells at the site of inoculation, and (4) the infiltration of leukemic cells as measured by weights of infiltrated organs. The results are shown in Tables II, III and IV. The tumor mass at the site of inoculation was well controlled, (Tables II and III) the duration of life was extended following a 6-day period of treatment with Compound A both

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1. Heilman, F. R., and Kendall, E. C., *Endocrinol.*, 1944, v34, 416.

2. Murphy, J. B., and Sturm, E., *Science*, 1944, v99, 303.

3. Law, L. W., and Speirs, R. S., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 226.

4. Diller, I. C., Beck, L. V., and Blanch, B., *Cancer Research*, 1948, v8, 581.

TABLE I.

Comparative Organ Weights and Hematologic Findings of Male and Female AK (RIL) Compound A Treated and Control Mice; Treated for 7 Days with Autopsy on the 8th Day. Body wt in g.

	Treated		Non-treated	
No. of mice	2	2	4	4
Mean age (days)	60	60	62	62
Mean body wt, at start	25.2	22.8	24.6	22.0
Mean body wt, at autopsy	25.8	24.5	25.6	22.9
Mean organ wt (% of autopsy body wt)				
Thymus	.020	.028	.248	.420
Spleen	.117	.114	.338	.363
Mesenteric lymph nodes	.032	.034	.155	.205
Liver	7.15	6.45	6.48	6.38
Brain	1.75	1.86	1.71	2.02
Heart	.911	.738	.797	.801
Kidney	.924	.698	.876	.723
Vesicular glands and prostates	.597		.790	
Testes and epidydimi	.520		.547	
Preputial	.366		.410	
Bulbo-urethral	.052		.092	
Uterus, ovaries, vagina		.391		.679
Submaxillary	.530	.426	.590	.424
Adrenal	.005	.010	.008	.015
Pituitary	.004	.007	.006	.009
Hematologic findings				
Mean total R.B.C. $\times 10^6$	8.2	9.4	10.7	11.1
" " W.B.C. $\times 10^3$	12.5	24.5	9.4	5.8
" % lymphocytes	8.0	3	57.6	59.2
" % monocytes	1.5	1	3.8	3.6
" % polymorphonuclear leukocytes	90.5	96	38.5	36.9
" % eosinophils	.0	.0	.1	.3

TABLE II.

Effect of Compound A on Lymphatic Leukemia. DBA Mice Treated for 6 Days Starting 2 Days After Tumor Transfer. Body wt in g.

	Treated tumor	Non-treated tumor	Non-treated non-tumor
No. of animals	3	3	5
Mean body wt at start	14.7	16.4	—
" body wt at 9 days	15.5	20.4	17.9
" tumor mass at 9 days in sq mm (L x W)	0	180.0	0
" days survival	15.4	10.7	—
Hematologic findings 7 days after tumor transfer			
Mean total R.B.C. $\times 10^6$	7.1	8.5	9.7
" total W.B.C. $\times 10^3$	9.1	13.5	7.1
" % lymphocytes	8.5	49.0	62.0
" % monocytes	0.2	5.5	5.5
" % polymorphonuclear leukocytes	91.3	45.2	32.4
" % eosinophils	.0	.3	.1

when given early (Table II) and late (Table IV) in the course of the disease, the mean total W.B.C. including the mean percent lymphocytes was lower (Tables II, III and IV) and spleen and thymus size was lower (Table III) in the Compound A treated groups.

The criterion for determining the response of the lymphosarcoma to Compound A was the mean tumor size. This tumor growth was well controlled for the short time of the present experiment (Table V).

TABLE III.

Effect of Compound A on Lymphatic Leukemia. DBA Mice Treated with 2 mg/day for 8 Days Starting the 1st Day After Tumor Transfer. Autopsy and Hematologic Readings on the 9th Day. Body wt in g.

	Treated tumor	Non-treated tumor	Non-treated non-tumor
No. of animals	3	3	3
Mean body wt at start	22	19	—
" body wt at autopsy	22	22	20.5
" tumor mass in sq mm (L x W)	0	144.0	0
Mean organ wt (% of body wt)			
Adrenal	.006	.009	.014
Spleen	.074	.164	.067
Thymus	.003	.014	.024
Hematologic findings			
Mean total R.B.C. $\times 10^6$	8.7	8.3	9.2
" total W.B.C. $\times 10^3$	5.2	17.7	5.6
" % lymphocytes	15.3	55.3	63.3
" % monocytes	3.2	5.0	4.8
" % polymorphonuclear leukocytes	81.5	38.7	31.9
" % eosinophils	.0	1.	.0

TABLE IV.

Effect of Compound A on Lymphatic Leukemia. DBA Mice Treated for 6 Days Starting the 7th Day After Tumor Transfer. Body wt in g.

	Treated tumor	Non-treated tumor
No. of animals	3	(6*) 3
Mean body wt, start	18.2	17.5
" body wt, 10 days	18.8	—
" days survival	14.8	10.7
Hematologic findings on 10th day after tumor transfer		
Mean R.B.C. $\times 10^6$	5.4	4.7
" W.B.C. $\times 10^3$	12.3	83.0
" % lymphocytes	12.8	57.3
" % monocytes	1.5	2.0
" % polymorphonuclear leukocytes	85.7	40.7
" % eosinophils	.0	.0

* 3 of 6 mice died of the leukemia before the 10th day and are not otherwise included.

Discussion. Lymphoid tumors were selected for study with the limited amount of Compound A available since there is a growing body of evidence showing that these tumors, as well as the normal lymphatic tissues of animals, undergo involution with increased adrenal cortical function. Reference has been made to the work of Heilman and Kendall, and others who have demonstrated tumor restraining activity of adrenal cortical extracts and Compound E. Recently evidence has been published showing adrenocorticotropic hormone (ACTH), presumably through stimulation of adrenal cortical function, also leads to the temporary regression of lymphoid tumors in man(5).

The quantitative problem is an important one. In the present experiments we have used at least four times the amount of Compound A that we would have been able to use of Compound E without causing serious disturbances or death of the animal. Speirs and Meyer have observed that a much larger dose, 25 γ, of Compound A is required to produce a reduction in eosinophil count similar to that obtained with 3 γ of Compound E(6).

Summary. The study provides evidence that Compound A (11-dehydrocorticosterone acetate) has pronounced biological activity. Among these is the ability to reduce the size of: (1) the adrenal glands, (2) tissues related to the lymphoid system and, (3) possibly the accessory reproductive organs. The life expectancy of mice bearing transplantable lymphatic leukemia P1534 was increased

5. Pearson, O. H., Eliel, L. P., Rawson, Rulon W., Dobriner, Konrad, and Rhoads, C. P., *Cancer*, 1949, v2, 943.

6. Speirs, R. S., and Meyer, R. K., *Endocrinol.*, 1949, v45, 403.

TABLE V.
Comparative Tumor and Organ Weight of Compound A Treated and Control C3H Lymphosarcoma Mice Treated for 6 Days Starting the 3rd Day; Autopsy the 9th Day. Body wt in g, tumor wt in mg.

	Treated tumor	Non-treated tumor	Non-treated non-tumor
No. of animals	3	3	3
Mean body wt at start	19.3	19.	—
" body wt at autopsy	20.0	23.7	19.3
" wt tumor mass	.0	1350	.0
Mean organ wt as % of body wt less tumor wt			
Spleen	.24	.74	.59
Mesenteric lymph nodes	.011	.030	.29
Thymus	.016	.093	.111
Adrenal	.007	.009	.011

and evidence of modification of the local lymphatic lesion was observed. Growth of the tumor mass of lymphosarcoma 6C3HED was prevented for a short experimental period.

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Role of the Pituitary in Growth of a Transplanted Rat Tumor.* (17879)

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The purpose of the present investigation is the study of the hypophyseal influence on the rate of growth of a transplanted rat tumor. This subject has received only a limited amount of attention in recent years, and as some of the earlier work is open to criticism regarding the completeness of hypophysectomy, we refer only to a more recent paper by Chamorro and Dobrovolskaia-Zavadskaia (1) for a bibliography on this subject.

Materials. Male rats of the Sprague-Dawley† strain were used throughout this investigation. The hypophysectomized animals were of the same weight range (80-90 g) as the controls at the time of the operation. The tumor used was the Walker Carcinosarcoma 256‡; Anterior Pituitary Powder, Parke-Davis & Co., No. H 1684E and whole dried Anterior Pituitary Lobes, Massone Institute, Buenos Aires.

Exp. I. Tumor implantation was made into the hypophysectomized rats 3 days after the hypophysectomy; the intact control animals were implanted at the same time. The animals were given a diet of Purina Laboratory Chow with tap water *ad libitum*. They were weighed twice weekly. The animals were sacrificed on the 14th day after tumor implantation, and the tumors removed and weighed immediately.

Exp. II. The conditions of this experiment were the same as for Exp. I with the exception that tumor implantation was delayed until 11 days after the surgical intervention, and that the animals were sacrificed on the 15th day following tumor implants.

Exp. III. Tumor implantation was made

* The expenses of this investigation were defrayed by the U. S. Vitamin Corp. of New York City, to whom our sincere thanks are offered.

1. Chamorro, A., and Dobrovolskaia-Zavadskaia, N., *C. r. soc. biol.*, 1945, v139, 614.

† Supplied via air freight by Hormone Research Laboratories, Inc., Chicago, Ill.

‡ We wish to thank Dr. K. Sugiura of the Sloan-Kettering Institute for making this tumor available to us.

TABLE I. (Exp. I).

Group	No. of animals	Surviving animals	Initial body wt (avg g)	Avg change body wt, %	Median tumor wt (g)
Hypo	10	10	78.1	— 5.6	0.895
Controls	11	11	91.3	+51.5	6.33

TABLE II. (Exp. II).

Group	No. of animals	Surviving animals	Initial body wt (avg g)	Avg change body wt, %	Median tumor wt (g)
Hypo	24	11	77.3	— 4.3	0.72
Controls	20	20	124.1	+45.0	3.30

TABLE III. (Exp. III).

Group	No. of animals	Surviving animals	Initial body wt (avg g)	Avg change body wt, %	Median tumor wt (g)
H	21	16	76.4	+13.5	2.905
A	21	13	77.9	+28.9	4.145
B	21	9	79.0	+28.6	3.69
Controls	20	20	93.2	+80.5	9.71

TABLE IIIa. (Exp. III).
Average Weight of Endocrine Glands (mg).

Group	Testes	Seminal vesicles	Adrenals	Thymus
H	217	7.5	20	248
A	710	28.0	23	285
B	666	21.0	18	241
Controls	2065	113.0	44	440

into the hypophysectomized rat 3 days after the operation, the intact controls being implanted at the same time. The animals were given a diet of Purina Laboratory Chow supplemented with canned dog food, whole milk powder, and whole wheat bread. They were given tap water containing 1% of sucrose *ad libitum*.

The hypophysectomized animals were divided into 3 groups: Group H received no injections, Group A received daily (5 inj. per week) subcutaneous injections of freshly dissolved Anterior Pituitary Extract A, Group B the same treatment as Group A, only with extract B. A total of 90 mg of each extract was given for the whole experimental period. The animals were weighed bi-weekly, and were sacrificed 15 days following the tumor implantation and the tumors, testes, seminal vesicles, adrenals, and thymus of each animal were removed and weighed immediately.

Ant. Pituitary Extract A. Ant. pituitary powder was extracted with 0.4 N NH_4OH in 60% ethanol (40 ml per g of powder). The soluble portion was diluted with 10 vol. of acetone, and acetic (glacial) acid was added to the point of flocculation. After standing overnight at room temperature, the precipitate was centrifuged off, washed with acetone and ether and dried *in vacuo*.

Extract B. The dried anterior pituitary lobes were extracted with 0.5 N NH_4OH (40 ml per g of lobes), with subsequent treatment as for extract A.

Discussion. Two criteria were used to determine the completeness of hypophysectomy in our test animals, namely, growth curves and testicular size. Cortis-Jones and Alt(2) report that when the testes of the operated rats (90-110 g weight) weighed over 500 mg 10-14 days after hypophysectomy, the removal of the gland was regarded as incomplete. In our experiments, the maximum weight of testes in untreated animals was 310 mg, which is satisfactory.

2. Cortis-Jones, B., Crooke, A. C., Henley, A. A., Morris, P., and Morris, C. J. O. R., *Biochem. J.*, 1950, v46, 173.

The results (Table I) of Exp. I show that hypophysectomy causes a very definite reduction in tumor growth, but does not completely suppress the growth of an implanted tumor. The tumors, apparently, grow to some extent independently of the pituitary gland; in this, we corroborate the results of the majority of the earlier workers on this subject. In Exp. II, tumor implantation was delayed until 11 days after the pituitary removal, in an effort to diminish the amounts of residual circulating hormones. This delay does not materially alter the picture (Table II).

Exp. III (Table III) confirms the results of Experiments I and II insofar as the growth-suppressing action of the pituitary is concerned. In addition, it may be stated that the enriched diet employed in this experiment exerted a growth-stimulating effect on the tumors. Comparing Group H of Exp. III with the operated animals of Experiments I and II, the body growth and tumor growth-promoting properties of an enhanced diet are quite evident. However, comparing Groups H, A, and B of Exp. III, the additional body and tumor growth of Groups A and B, over Group H, can only be due to the pituitary extracts administered. The tumor growth-inhibiting action of the pituitary removal may, therefore, be partially counteracted by injection of anterior pituitary extracts.

The only hormonal principle clearly demonstrable in our anterior pituitary extracts was the gonadotrophic hormone, as is evident from Table IIIa. In this connection, the relation between testes size and tumor size may be of significance.

Summary. Hypophysectomy in rats definitely, but incompletely, suppresses the growth of a transplanted tumor. It appears possible that a hormone, or hormones, of the pituitary are required for stimulation of tumor growth as evidenced by the effects of pituitary gland removal and the partial results of the administration of certain anterior pituitary extracts.

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Blood Flow Determinations and Cardiodynamic Effects of a Venous Shunt in Pulmonary Hypertension.* (17880)

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The following study was prompted by the recent report of Bland and Sweet(1) in which a surgical anastamoses of the azygous vein and a branch of the right pulmonary vein was established for relief of pulmonary venous hypertension in certain instances of mitral stenosis. Marked clinical improvement was noted in 3 of the 5 patients operated, indicating that such a venous shunt was presumably capable of relieving the high pressure in the pulmonary area. It was our purpose to test the capacity of this type of shunt with various acute procedures designed to increase pulmonary pressure and if possible, note the effects of the venous shunt on the cardiodynamics of such animals.

Method. The 10 dogs used in these experiments were anesthetized with sodium barbital I.V., 300 mg/kg. The chest was opened in the mid-sternal line, the pericardial sac was incised, and its edges were sewn to the thoracic walls in such a manner as to form a cradle for the heart. Respiration was maintained by use of alternating positive air pressure. The azygous vein was ligated 2 to 3 cm from the superior *vena cava*; and cannulae were inserted, one in the azygous vein proximal to its ligation and one in a large

* This work was supported by a grant from the American Heart Assn.

1. Bland, E. F., Sweet, R. H., *J.A.M.A.*, 1949, v140, 1259.

tributary of the right pulmonary vein. These cannulae were connected through a Gregg-Shipley rotameter(2) in such a manner as to shunt blood from a tributary of the pulmonary vein to the azygous vein. Blood flow through the shunt could then be measured. An 8% solution of Chlorazol Fast Pink dye, 2 cc per kilo, was used as the anticoagulant. Aortic pressure curves were recorded by inserting a sound connected to an optical manometer(3) through the left carotid artery. The cardiac output was determined by the pressure pulse method and is expressed per M^2 of the body surface(4). Pressures in the right ventricle were measured by inserting a sound through the right jugular vein into the right ventricle. In the experiments in which the left pulmonary artery was occluded, the artery was freed by blunt dissection and clamped with a rubber sleeved curved hemostat. Stenosis of the pulmonary artery was obtained by passing a ligature around the artery just distal to the pulmonary conus and drawing the loose ends of the ligature through a short, 3 to 4 cm, glass tube. The tube was pressed against the artery as the ligature was tightened, and the 2 loose ends were then clamped at their exit from the tube.

The method used to produce experimental mitral stenosis was suggested in a paper by Katz and Siegel(5). A suture was anchored posteriorly near the interauricular groove and above the auriculo-ventricular groove. The suture was then looped through the auricular tissue nearer the hilar structures of the left lung. Anteriorly a suture was anchored at the base of the left auricular appendage near its medial aspect; the anterior suture, in a like manner, was looped through the auricular tissue nearer the hilar structures. When desired, constriction of the left atrium could then be effected by threading the 2 loose suture ends through an aneurysm needle and snugging the aneurysm needle against the auricular wall by traction on the sutures. The sutures were then clamped in place with a hemostat at their exit through the aneurysm needle. To determine if flow through the shunt could be further increased, following constriction of the left atrium, a 6% solution of acacia was infused rapidly via a femoral vein in one dog. Controls with shunt open were run prior to obstruction of outflow through the pulmonary arteries or constriction of the left atrium.

Results. Effects on blood pressure. Changes in aortic pressure were only slight, on the order of ± 2 to 10 mm Hg, in those dogs in which occlusion of the left branch of the pulmonary artery was performed. There was a variable effect on those dogs with constriction of the left atrium. A significant rise in both systolic and diastolic pressures was obtained in one animal. See Table I. One other animal which, in addition, received acacia intravenously demonstrated a parallel rise in arterial pressure. In other animals with constriction of the left atrium there was little change in the arterial pressure.

Pressure in the right ventricle. The initial tension in the right ventricle usually was not altered. There was a significant rise of initial tension in only one animal (Table I).

Blood flow through the shunt. Blood flow through the shunt without attempts to produce pulmonary hypertension, varied from 37 cc to 84 cc per minute per M^2 of body surface. The rate of flow could be increased from 2 to 3 fold either by occlusion of the left branch of the pulmonary artery or by constricting the left atrium.

Heart rate. Following obstruction to pulmonary arterial flow or constricting the left atrium, the heart rate slowed on the order of 10 to 18 beats per minute.

Cardiac output. The cardiac output was diminished in each animal following either restriction of flow through the pulmonary arteries or constriction of the left atrium. There seemed to be no appreciable effect on cardiac output whether the shunt was open or closed following the above procedures.

Discussion. Bland and Sweet pointed out that too large a shunt would be undesirable

2. Crittenden, E. C., Jr., and Shipley, R. E., *Rev. Scient. Instruments*, 1944, v15, 343.

3. Hamilton, W. F., Brewer, G., and Brotman, I., *Am. J. Physiol.*, 1934, v107, 427.

4. Hamilton, W. F., and Remington, J. W., *Am. J. Physiol.*, 1947, v148, 14.

5. Katz, L. N., and Siegel, M. L., *Am. Heart J.*, 1931, v6, 672.

TABLE I.
Results in One Animal with Constriction of Left Atrium.

Procedure, shunt open	Aortic pressure, S/D	Initial tension of right ventricle, mm/Hg	M^2 body surface: Blood flow through shunt, cc/min.	M^2 body surface: Cardiac output, cc/min.	Cardiac rate
Control	138/112	22	37	3600	186
Shortly after constrict.	154/122	24	95	3300	186
30 min. constrict.	160/126	28	126	3300	168
Constrict. removed	128/ 98	22	55	3900	174
Shortly after constrict. removed	134/ 96	18	34	4030	174

(1). Shunting of blood through a large extracardiac shunt might lower pulmonary vascular pressure to such an extent that it would be insufficient to overcome the obstructed inlet to the left ventricle, and the possibility of overburdening the right ventricle with increased venous return might exist. The capacity of the shunt in our acute experiments could be increased up to 8% of the cardiac output. An effort to further increase the capacity of the shunt by rapidly infusing acacia intravenously, thereby increasing venous return and overloading the right ventricle, was not successful. One cannot conclude from acute experiments of this type that the maximum shunt flow was obtained. Although the cardiac output was diminished, there was no conclusive evidence that the presence of the venous shunt interfered with the filling of the left ventricle. Evidence of right ventricular strain by virtue of a rise in initial tension was found in only 2 of the animals. This was also noted to be variable by Katz and Siegel in their study (4). In addition, the increase of venous return, a consequence of the presence of the shunt, gave no evidence that an added burden was placed on the right ventricle. In this regard the effects over a prolonged period remain to be elucidated.

Conclusions. 1. In acute experiments producing increased pulmonary vascular pressure in dogs, the capacity of a pulmonary vein-azygous vein shunt was found to be up to 8% of the total cardiac output.

2. There was no consistent evidence that the presence of the shunt produced or added to the strain on the right ventricle or interfered with the filling pressure of the left ventricle.

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Maternal Nutrition and Hydrocephalus in Newborn Rats.* (17881)

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It is well known that female rats reproduce readily on synthetic diets, but Richardson and Hogan(1) reported that 1.7% of the young in their colony developed hydrocephalus, due to a deficiency in the maternal diet. O'Dell, Whitley and Hogan(2) observed that when folic acid was added to the diet used by Richardson and Hogan the incidence of

* Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station, Journal Series No. 1212. This investigation was supported in part by a grant from the U. S. Public Health Service.

1. Richardson, L. R., and Hogan, A. G., *J. Nutrition*, 1946, v32, 459.

2. O'Dell, B. L., Whitley, J. R., and Hogan, A. G., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 272.

hydrocephalus was sharply reduced. This observation confirmed the nutritional origin of the abnormality and suggested that the incidence would be increased if the folic acid deficiency were sufficiently severe. The object of this paper is to describe the procedure, and the results obtained, in our attempt to limit the amount of folic acid that was available to the experimental animals.

Experimental. In previous studies all rats had consumed synthetic diets, with casein as the source of protein. However, it was observed in a parallel study not described in this report, that there were a few cases of hydrocephalus in the young of females on a diet in which the protein was supplied by soybean oil meal. The reproductive record was excellent in all other respects and it was decided to use both the casein diet, No. 1953, and the soybean oil meal diet, No. 2087, in further studies of hydrocephalus. The incidence of the abnormality remained distressingly low and it was assumed that the rats had obtained sufficient folic acid, as a result of intestinal synthesis, to prevent all but a few cases of hydrocephalus. In an effort to reduce the amount of available folic acid a folic acid antagonist, crude methylfolic acid†, was added to the soybean oil meal diet, No. 2087. One of the new diets, No. 2115, contained 1 mg of the antagonist per 100 g. The other, No. 2116, contained 2 mg. The results obtained with these diets were approximately the same and are all ascribed to No. 2115 to simplify the presentation. These new rations, of which 2115 is an example, were consumed by all rats during the period included in this report. During the pre-experimental period some of them had received (a) the soybean oil meal diet, No. 2087, (b) others had received the casein diet, No. 1953, and (c) another group had received the stock diet, on which our rat colony is maintained. The more important diets are described in Table I and the results summarized in Table II. The incidence of hydrocephalus, at least for some months, depended on the diet the females had consumed before the folic acid antagonist was included in the diet. For that reason the results obtained with rats that consumed the soybean oil meal diet before the antagonist was added will be discussed first.

† Generously supplied by T. H. Jukes of the Lederle Laboratories, Pearl River, N. Y.

TABLE I.
Composition of Experimental Diets.

Ration No.	1953	2087
	g	g
Casein (Vit. test)*	30	—
Soybean oil meal	—	70
Cerelose	52	22
Wood pulp	3	—
Lard	10	4
Salts†	5	4
Added vitamins,‡ both diets		
		mg
Thiamine HCl		1.6
Riboflavin		1.6
Pyridoxine HCl		1.6
Ca-pantothenate		4.
Choline chloride		100.
Biotin		0.02
Alpha tocopherol		1.
2-methyl-1,4-naphthoquinone		1.
Vit. A		2000 I.U.
Vit. D		285 I.U.

* Nutritional Biochemicals Corp., Cleveland, O.
† Richardson and Hogan(1).
‡ Vit. A and D were supplied in the form of oleum percomorphum, Mead Johnson & Co. All other vitamins were supplied through the courtesy of Dr. D. F. Green, Merck & Co., Rahway, N. J.

a. *Soybean oil meal diet during the preperiod.* The first hydrocephalic rat in this group was born 19 days after the folic acid antagonist was added to the diet of the mother. On the 29th day after the change a litter of 13 was born, all of which were hydrocephalics. If this litter is excepted, the number of hydrocephalics per litter has varied from 0 to 6. Over half of the litters contained at least one hydrocephalic, and over 20% of all young born had hydrocephalus. One female bore 19 young with 14 hydrocephalics, distributed among each of her 4 litters. Another bore 5 litters with 23 young. There were 3 hydrocephalics distributed between 2 litters.

b. *Casein diet during the preperiod.* It will be observed in Table II that the incidence of hydrocephalus was quite low in the 1st and 2nd litters borne by females in this group, and there was a definite increase in their 3rd litters. There were only three 4th

TABLE II.
Maternal Nutrition and Incidence of Hydrocephalus in the Offspring.

Days on exp.	Litter order	No. of litters: Total	No. of litters: With hydrocephalic young	No. of young: Total	No. of young: Born dead	No. of young: Hydrocephalic	No. of young: Survived 28 days
A. Ration 2087 before transfer to experimental diet							
19–68	1	12	6	115	0	25	32
55–135	2	12	8	88	6	18	13
83–144	3	12	7	71	3	18	18
111–151	4	6	4	41	2	10	10
139	5	1	1	5	1	1	0
B. Ration 1953 before transfer to experimental diet							
14–78	1	13	1	115	4	1	78
68–144	2	13	1	78	5	2	55
103–155	3	7	3	55	5	5	7
139–158	4	3	2	12	0	3	0
C. Stock ration before transfer to experimental diet							
58–102	1	10	1	79	4	1	20
88–109	2	4	0	27	3	0	1

litters but it is significant that the percentage of hydrocephalics rose to practically the same level as was reported in Section A.

c. *Stock diet during the preperiod.* The last group has been under observation a shorter time than the others and was made up of females that were transferred directly from the stock diet to Ration 2115. In 109 days after the change in diet 14 litters were born, containing 106 young, with one hydrocephalic.

During the period covered in this paper, all cases of hydrocephalus recorded were detected at birth and none developed later. If distortion of the skull is too slight for a sure diagnosis, doubtful cases are usually diagnosed with certainty by observing the intensity of the transmitted light when the head is held in a strong beam of light in a line with the eye of the observer. Practically all of the hydrocephalics have died within 3 or 4 days and only 3 out of a total of 72 have survived as long as 3 weeks.

Discussion. There is no doubt that the incidence of hydrocephalus can be greatly increased by adding the methylfolic acid antagonist to the diet, but the cause of the increase is as yet uncertain. It may be relatively simple, and merely due to a more severe deficiency of folic acid. On the other hand it is conceivable that methylfolic acid has some specific toxic effect and hydrocephalus is the result of a structural or biochemical derangement that is unrelated to a folic acid deficiency. Gillman, Gilbert and Gillman(3) injected female rats with trypan blue, and observed that 7.3% of their young were hydrocephalics. We had no difficulty in confirming this observation. One could assume that the initial damage was the same whether trypan blue or methylfolic acid was administered to the females.

Another point that deserves some consideration is the long delay in the appearance of hydrocephalic young when the females were transferred from the casein diet to the soybean oil meal diet which contained a folic acid antagonist. The casein diet did not contain folic acid and one would not suppose that a long period would be required to reduce the tissue reserves to a critical level. It may be that still another nutrient is involved in the development of hydrocephalus, and we are considering the possibility that it is vitamin B_{12}. The stock diet undoubtedly contains it, and according to our microbiological assay the casein used contained 1.8 μg %. The soybean oil meal has not been assayed but we know it is a poor source of this vitamin. However, the possibility is not excluded

3. Gillman, J., Gilbert, Christine, and Gillman, T., *S. Afr. J. Med. Sci.*, 1948, v13, 47.

that an unrecognized nutrient is implicated.

Hydrocephalus has been reported in various animal species though it is too rare to be of any practical significance in livestock production. It occurs in human infants, and though uncommon it is of some consequence. No satisfactory explanation of these cases has been advanced but it seems plausible now that many at least are the result of a nutritional deficiency. This possibility raises a question that deserves serious consideration; is there any wide variation in the severity of the symptoms. Up to the present hydrocephalus seems to be an all or none phenomenon but an attempt will be made to determine whether mild cases occur that escape detection by the method used in the past. The possibility of tissue or functional damage, with no gross symptoms of injury, deserves serious study.

Summary. Female rats were supplied with an experimental diet that contained soybean oil meal as a source of protein, and a vitamin mixture that included all recognized vitamins except ascorbic acid, niacin, folic acid and B_{12}. The incidence of hydrocephalus in the young was less than 1%. When folic acid antagonist was added to this diet the incidence of hydrocephalus rose to 20%. The type of diet consumed during the pre-experimental period determined the amount of time that elapsed, during the experimental period, before hydrocephalus appeared in the young.

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The Cockroach as an Experimental Vector of the Virus of Spontaneous Mouse Encephalomyelitis (Theiler).* (17882)

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The natural modes of transmission for the polio-encephalomyelitis group of viruses are not understood(1,2). A commonly accepted epidemiological hypothesis encompasses a fecal-oral spread and the alimentary tract as the portal of entry. In support of this hypothesis is the finding of virus in the intestinal tract, stools, sewage, and flies. The demonstration(3) that virus persists in non-biting flies for days, and possibly multiplies, suggests that members of the polio-encephalomyelitic group of viruses escape the intestinal-oral carrier chain in their natural mammalian hosts to utilize arthropods for the maintenance and passage of virus to new hosts. Attempts to incriminate mosquitoes(4-6) have been unsuccessful. Lice(7) and fleas(8,9) were found incapable of transmitting the virus of human poliomyelitis. Since the capacity of cockroaches† for the maintenance and transmission of viruses has been little

* Aided by a grant from the National Foundation for Infantile Paralysis.

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TABLE I.
Recovery of Encephalomyelitis Virus, GD VII Strain from Cockroach Feces.

		Results of the injection of a fecal suspension derived from Roaches fed virus									Normal roaches
Days after single meal		0	1	2	3	4	5	6	7	8	1
Mouse	1	0/2*	2/2	2/2	2/2	2/2	2/2	2/2	0/2	0/2	0/2
Passage	2†	0/2	2/2	2/2	2/2	2/2	2/2	2/2	0/2	0/2	0/2

* Denominator signifies number of mice injected intracerebrally, 0.03 ml. Numerator signifies number that died of encephalomyelitis.

† Brain tissue from the mice of passage 1 was employed as the inoculum for passage 2.

explored, an experimental study was designed to learn whether a representative of the family *Blattidae* could acquire, maintain, and excrete a member of the polio-encephalomyelitic group of viruses. Cockroaches were selected as arthropods especially worthy of study because in many parts of the world the intimacy and constancy of the association of cockroaches with the food and excreta of man and animals rivals closely that of flies. Moreover, the nocturnal habits, gregariousness, and longevity of the cockroach operate to provide opportunities not readily available to flies for the acquisition of virus from excreta and for its transfer to food.

It is the purpose of this preliminary report to record the findings which were obtained when the American cockroach, *Periplaneta americana,* was investigated for its capacity to act as the intermediary for the maintenance and excretion some days later of the virus of mouse encephalomyelitis, GD VII strain (Theiler) (10).

Experimental. Adult cockroaches (*Periplaneta americana)* were strapped to corks with adhesive tape. Each cockroach was given by means of a tuberculin syringe with a blunt needle a single feeding of 0.2 ml of a 10% suspension of brain tissue derived from a mouse moribund of spontaneous mouse encephalomyelitis, GD VII strain. The diet thereafter consisted of fox chow and water. Fecal specimens representative of a 24-hour period were collected daily and stored at -20°C until tests for the presence of virus were made. Each 24-hour fecal specimen in preparation for inoculation was made up separately by trituration in a mortar with normal physiological saline to yield a 1% suspension, treated in an amount per ml of suspension with penicillin, 500 units, and streptomycin, 100 μg per ml, mixed, allowed to stand at room temperature for 30 minutes, and centrifuged at 700 r.p.m. for 10 minutes. The supernatant fluid was employed as the inoculum for the injection intracerebrally, 0.03 ml, of normal 21-day-old Swiss albino mice. The control consisted of fecal material obtained from normal cockroaches and made ready in a similar manner for transfer to normal mice.

Results. The results of one set of experiments are shown in Table I. In these experiments the 24-hour fecal specimens for each of 6 consecutive days when tested as a 1% suspension was shown to contain sufficient virus to paralyze and kill mice in from 2 to 4 days after its intracerebral injection. The test mice for the seventh day remained normal. The brain tissue in 1% suspension from dead mice was lethal on passage to normal mice.

In contrast to these findings, the mice that were utilized to test similarly prepared fecal suspensions from normal cockroaches showed no evidence of infection during a 14-day period of observation. Bacteriological control studies of all mouse brains eliminated the

† Five species of cockroach of cosmopolitan distribution are commonly associated with man: *Blattella germanica,* the small German roach or "croton bug"; *Blatta orientalis,* the larger oriental roach; *Periplaneta australiasiae,* the Australian roach; *Supella supellectilium,* the tropical roach; and *Periplaneta americana,* the large-winged American cockroach.

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possibility of bacterial infection.

Three more sets of experiments were carried out by following the same procedure. These experiments confirmed fully the results described above and gave further information since in one instance virus was recovered from the 24-hour fecal specimen representative of the seventh day after a single feeding of virus.

Discussion. Under the experimental conditions described in the present paper, cockroaches fed a single meal known to contain the virus of spontaneous mouse encephalomyelitis, GD VII strain, excreted daily over a period of as long as 7 days sufficient virus to kill on intracerebral injection the test recipient normal mice. Thus, it is possible that this species of cockroach, and others, in the natural process of feeding on mammalian excreta may acquire virus from the host carrier for later transfer by contamination of food. From the available data it is impossible to say whether these findings have any practical implication in the epidemiology of mouse encephalomyelitis or of other members of the polio-encephalomyelitis group of virus diseases. It is surprising that natural or experimental evidence to suggest a role for cockroaches in the dissemination of virus diseases is limited to the experimental findings of Hurlbut(11) which appeared at the time this manuscript was in preparation. He found that the injection of human poliomyelitis virus, Lansing strain, into the hemocoele of the cockroach, *Periplaneta americana,* made it possible 15 days later to demonstrate virus by the trituration of the whole cockroach and its passage to normal mice. Attempts to demonstrate virus in roach feces and in roach eggs were unsuccessful. However, the possibility that cockroaches can operate to transmit pathogenic microorganisms other than viruses has been established for bacteria under experimental and natural conditions (12-14).

Summary. In experiments in which cockroaches, *Periplaneta americana,* were fed a single meal containing the virus of mouse encephalomyelitis, GD VII strain, proof was obtained that the test cockroaches daily over a period of as many as 7 days excreted sufficient virus to kill test mice. Control experiments showed that the lethal effect was from infection by the virus of mouse encephalomyelitis, GD VII strain, and not from extraneous bacterial or viral infection.

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Influence of Diphenhydramine on Blood Pressure Response to Epinephrine in the Dog under Adrenergic Blockade. (17883)

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A variety of substances have been shown to potentiate the vasopressor effect of epinephrine: *vis.* diphenhydramine·HCl, cocaine, ergotamine, curare, dibutoline, tetraethylammonium chloride (T.E.A.) and a number of ethylene diamine derivatives(1,2,3,4,5,6,7, 8). Two theories have been suggested for

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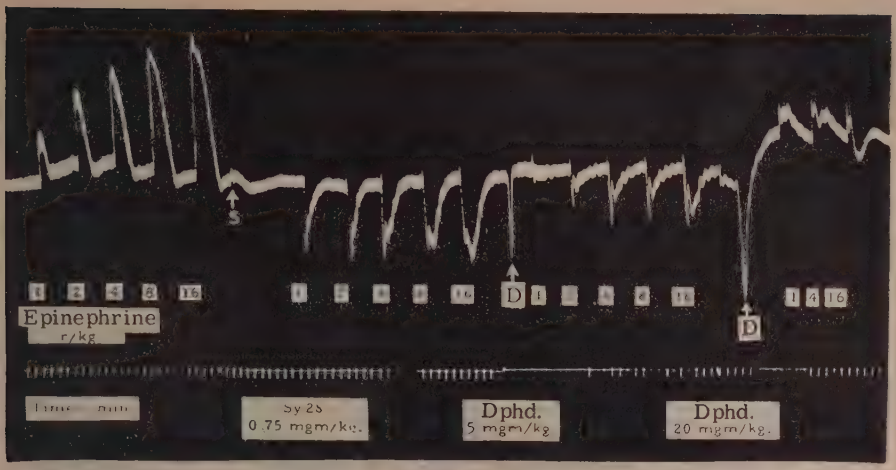

FIG. 1.
Influence of SY-28 and diphenhydramine on the effect of epinephrine on blood pressure in the dog. Pentobarbital anesthesia.

their mode of action: (a) inhibition of the enzymatic destruction of epinephrine, and (b) increase of permeability of sympathetically innervated cells(9,10). However, direct experimental evidence to support these hypotheses is lacking. In the case of T.E.A., the increased vasopressor effect of epinephrine is said to be due to its ganglionic blocking action abolishing the compensatory reflexes (11). In a preliminary report(12), it was mentioned that diphenhydramine can convert the "epinephrine reversal" of blood pressure to a pressor response. In this communication, data are presented to show the influence of diphenhydramine and some other agents on the vascular action of epinephrine and arterenol in dogs under adrenergic blockade. In light of these findings, the action of diphenhydramine in reversing the vasodepressor and in potentiating the vasopressor response to sympathomimetic agents will be elucidated.

Experimental. Dogs under pentobarbital anesthesia were used in our experiments. Blood pressure was measured from the carotid artery; injections were given in the femoral vein.

Results. The effect of diphenhydramine on the "epinephrine reversal" of blood pressure is shown by the kymographic recordings in Fig. I. The dog was injected at 5 minute intervals with increasing doses of epinephrine, followed by 0.75 mg/kg of N-(2-bromoethyl)-N-ethylnaphthalenemethylamine · HBr (SY-28). Ten minutes later the epinephrine injections were repeated. Being an adrenergic blocking agent, SY-28 reverses the vasopressor effect of epinephrine. Five milligrams per kilo of diphenhydramine was then injected. Subsequently the vasodepressor effect of epinephrine was first decreased, then converted to a pressor effect after a total dose of 20 mg/kg of diphenhydramine · HCl. Similar results were obtained for the vascular action of arterenol under the influence of diphenhydramine and SY-28. The vasopressor response to arterenol can be completely reversed by SY-28 (1 mg/kg); the maximal

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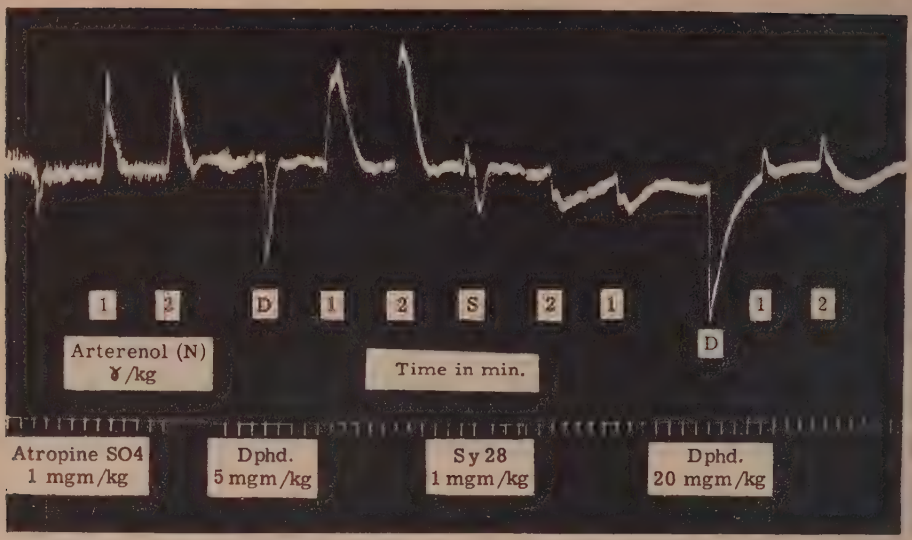

FIG. 2.
Influence of SY-28 and diphenhydramine on the effect of arterenol on blood pressure in the dog. Pentobarbital anesthesia.

vasodepressor effect is, however, only about 1/5 that of epinephrine (Fig. 2).

A number of compounds possessing the pharmacological properties of diphenhydramine were also investigated. The results are given in Table I. One salient feature revealed by the data is that compounds which reverse the vasodepressor response are also capable of potentiating the vasopressor response to epinephrine. Cocaine, diphenhydramine and ergotamine have been shown likewise to produce potentiation of the vasopressor action of arterenol(13,14). In agreement with the observation of others(15,16,17,18) we found ergotamine in pentobarbitalized dogs potentiated, in etherized animals reversed the vasopressor effect of epinephrine. It converts the vasodepressor response following N-isopropyl arterenol to a vasopressor effect in pentobarbitalized dogs. Resembling ergotamine, diphenhydramine (30 mg/kg) reduces the vasodepression produced by 1-2 μg per kilo of N-isopropyl arterenol and in some dogs converts it to a pressor effect.

Discussion. How diphenhydramine reverses the vasodepressor response to epinephrine is not understood. The anticholinergic, antispasmodic and antihistaminic properties of diphenhydramine are not responsible for this action, for atropine, papaverine and 2-n-propoxy-4, 6-diamino-s-triazine are ineffective in reversing the vasodepressor effect of epinephrine. The reversal of the vasodepressor response cannot be due to an increase in elimination of SY-28 in the presence of diphenhydramine or due to competitive inhibition on the same receptors upon which SY-28 acts. If either were the case, there should be expected a graded effect to epinephrine at different dose levels. Instead, as indicated in Fig. 1, after SY-28 and diphenhydramine the vasopressor effects of different doses of epinephrine are the same.

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TABLE I.
Reversal of Vasodepressor Effect of Epinephrine on Dog's Blood Pressure.
*SY-28 (Sympatholytic) = 0.75 mg/kg
Epinephrine = 1 γ/kg

	Compound	mg/kg I.V. (total dose)	Effect reversal
1.	Diphenhydramine†	10	Partial
		30	Complete
2.	P-methyl substituted derivative of diphenhydramine†	10	Partial
		30	Complete
3.	Cocaine†	20	Partial
		50	Complete
4.	Tripelennamine†	10	Partial
		45	Complete
5.	Pyranisamine maleate†	15	,,
6.	d-Tubocurarine†	0.25	,,
7.	Dibutoline • SO_4†	10.0	,,
8.	Ergotamine†	0.2	,,
9.	T.E.A.†	5	No
		20	Partial
10.	Atropine	35	No
11.	Papaverine	5	,,
12.	2-N-propoxy-4,6-diamino-s-triazine	105	,,

* N-(2-Bromoethyl)-N-ethyl-naphthalenemethylamine • HBr.
† Compounds which also potentiate the vasopressor effect of epinephrine.

This same rise of blood pressure may be due to the effect of epinephrine on the heart(19).

The action of diphenhydramine on the vasodepressor response to epinephrine can neither be explained by the increase of permeability of sympathetically innervated cells nor by an inhibition of the enzymatic destruction of epinephrine, for if so, a greater depressor response to epinephrine should be expected. The increase of the vasopressor effect of epinephrine by diphenhydramine may be through its antagonistic action on the vasodepressor mechanism in response to epinephrine. Since the blood pressure changes by epinephrine represent a composite picture of both the pressor and the depressor response(20), the results in Fig. 1 and 2 may be interpreted as follows: SY-28 blocks the vasopressor receptors while diphenhydramine antagonizes the response of the vasodepressor effectors. With sufficient quantities of the two, the action of epinephrine (or arterenol) on the sympathetic neuro-effectors (both vasoconstrictor and vasodilator) may be completely inhibited; the blood pressure response then is principally due to its chronotropic and inotropic action on the heart, as indicated by the last portion of the graph.

With the information now available it is not possible to state the site of action of diphenhydramine on the vasodepressor mechanism whether the peripheral vasodilator receptors or the central nervous pathway. The various agents which reverse the vasodepressor effects of epinephrine and arterenol may do so by acting on the different sites of the neurovascular system. The fact that they also potentiate the pressor effect of epinephrine and arterenol suggests that potentiation of the vasopressor response may be due to an inhibition on the vasodepressor effectors by these substances.

Conclusion. Diphenhydramine was found to convert the vasodepressor effect of epinephrine and arterenol in the dog under adrenergic blockade with SY-28 to a vasopressor effect. The para-methyl substituted derivative of diphenhydramine as well as cocaine, tripelennamine, pyranisamine maleate, d-tubocurarine, dibutoline and ergotamine can produce the same effect. Atropine (anticholinergic), papaverine (antispasmodic) and 2-N-propoxy-4,6-diamino-s-triazine (antihistaminic) are ineffective. An inhibition of

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the vasodepressor mechanism is suggested as a possible mode of action of these compounds on the reversal of the vasodepressor response and the potentiation of the vasopressor response to epinephrine and arterenol.

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Response of Circulating Eosinophils to Nor-Epinephrine, Epinephrine and Emotional Stress in Humans. (17884)

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The diminution of the number of circulating eosinophils after subcutaneous injection of epinephrine is believed to be due to an increased secretion of adreno-cortical glucocorticoids, elicited by a discharge into the blood of adrenocorticotrophic hormone(1,2) which, in turn, constitutes a typical reaction to injected or secreted epinephrine(3,4). On the other hand, it is becoming increasingly apparent that l-nor-epinephrine plays a prominent role in the manifestations of sympathetic stimulation as the specific chemical neuro-transmitter of the sympathetic system (5) and as a secretory by-product of the adrenal medulla(6,7). It seemed of interest therefore to investigate the following questions: (a) Does nor-epinephrine exert an analogous effect upon the pituitary-adrenocortical system as its methylated homologue epinephrine? (b) Are the neurovegetative phenomena of emotional stress attributable mainly to discharges of epinephrine from the adrenal medulla or to neuro-secretory discharges of nor-epinephrine?

Methods. Twelve patients with various disorders but not acutely ill, were tested to compare the response of the circulating eosinophils to equal amounts (by weight) of subcutaneously injected natural l-epinephrine* and synthetic l-nor-epinephrine† (0.25 mg of each). Blood samples from the finger tip were taken early in the morning for the initial eosinophil counts and the injections were administered immediately thereafter. Four hours later the eosinophil counts were repeated. No nourishment was taken preceding and during the tests. In 24 healthy medical students in the fasting state, finger tip blood samples for eosinophil counts were taken in the morning, 5 to 20 minutes before the students were to begin their mid-year examinations. Blood pressure and pulse rate were also recorded (after only a few minutes rest in sitting position). Several weeks later when no examinations were in sight the same students were again tested in the same manner. In all instances the direct eosinophil counts were carried out according to the method employed by Forsham *et al.*(2). However, the staining method of Randolph(9), as modified by Henneman *et al.*(10), was used in-

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* Adrenaline hydrochloride Parke & Davis was used, which contains besides l-epinephrine an estimated 10-19% admixture of l-nor-epinephrine(8).

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† We are indebted to Dr. M. L. Tainter of Sterling-Winthrop Research Laboratories for l-arterenol bitartrate (l-nor-epinephrine).

stead of the eosin acetone stain. The eosinophils were counted between 20 and 60 minutes after dilution of the blood samples.

Results. There was a striking difference between the effects of l-epinephrine and l-nor-epinephrine upon the eosinophil count in that the average decrease due to epinephrine was 61% (range: 41% - 84%) while analogous doses of nor-epinephrine caused an average fall of only 9% (range: 0% - 24%).

The average eosinophil count of the 24 students during a non-stress period was 128 per mm^3. Under emotional stress before examination it was 66 per mm^3. Thus, there was an average difference of -48%. Only in one case were the 2 counts identical. The greatest individual difference was -89%.

The average systolic blood pressure and heart rate were higher in the state of tension while the average diastolic pressure remained unchanged: 137/82 mm Hg and 96 beats as compared with 128/81 mm Hg and 82 beats.

Discussion. The relatively weak eosinophil-reducing effect of l-nor-epinephrine, in contrast to that of l-epinephrine, suggests a considerably weaker stimulating action of the former on the ACTH and gluco-steroid secretion. This assumption seems to be supported by recent animal experimentation: The doses of nor-epinephrine which were required to reduce the ascorbic acid content of the rat adrenal (criterion of gluco-corticoid secretion) (4) were found to be several times larger than the doses of epinephrine which sufficed to elicit an equal ascorbic acid depletion of the rat adrenal(11,12). As far as the marked eosinophil-reducing influence of emotional stress, demonstrated by the present data, is concerned, this observation makes it probable that epinephrine discharges from the adrenal medulla are largely, if not entirely, responsible for the accompanying neurovegetative phenomena. This is also suggested by the fact that the emotional cardiovascular reactions follow the pattern of epinephrine action (elevation of systolic pressure; little, if any, participation of the diastolic pressure; cardiac acceleration and palpitations), while nor-epinephrine injection causes a marked rise of both systolic and diastolic pressures with bradycardia and only a minimum of subjective sensations(13,14, 15,16).

A simultaneous discharge of some nor-epinephrine from both the adrenal medulla and post-ganglionic sympathetic fibres during emotional stress cannot be excluded but the present observations do not supply any positive evidence for it.

Summary. In human subjects the effectiveness of l-nor-epinephrine in reducing the circulating eosinophils was found to be only about one-sixth of that of epinephrine.

A marked diminution of the eosinophil count was observed in students in a state of emotional tension. This and the pattern of the accompanying cardiovascular reactions suggest that the neurovegetative manifestations under emotional stress are essentially caused by a discharge of epinephrine from the adrenal medulla. However, a certain degree of participation of neuro-secretory nor-epinephrine discharges cannot be entirely excluded.

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Radioautography of Cerebral Tumors Employing P^{32}.* (17885)

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(Introduced by A. B. Hastings)

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Radioactive phosphorus, given intravenously to patients with brain tumors, reaches concentrations in the tumor tissue which is ordinarily from 5 to 100 times as high as that in the normal cerebral tissue(1). A remarkable variation in P^{32} activity in adjacent regions of the same tumor was early recognized. For example, Geiger-Mueller counting of biopsy specimens provided crude but dramatic evidence that necrotic areas in a malignant glioma might take up only 0.1% as much P^{32} as regions of rapid growth(1). More precise correlation of these variations in the rate of uptake of P^{32} with microscopic appearance has been difficult. The heterogeneous character of certain gliomas makes it impossible in many cases to obtain specimens of uniform histologic pattern sufficiently large for assay by Geiger-Mueller counting. Since the grains of a photographic emulsion provide, in effect, a mosaic of minute "counters", a radioautographic technic permits the assay of P^{32} in each area of a tissue section, at the microscopic level. This communication describes the use of such a technic in order to secure a map of the varying uptake of radioactive phosphorus in brain and brain tumor as it is related to histologic pattern.

Method. Brain and brain tumor tissue have been obtained from patients in whom radioactive phosphorus has been used for the localization of cerebral tumors by means of a probe Geiger-Mueller counter(2,3). A single intravenous dose of from 0.5 to 4.0 millicuries of carrier free radioactive phosphate ion is given to presumptive brain tumor patients preferably from 12 to 72 hours before craniotomy. At operation an attempt is made to secure specimens of tumor for microscopic autography with a minimum of trauma. Control samples of normal gray and white matter are obtained when removal of overlying or adjacent cerebral tissue is necessary in order to expose the tumor. Samples from regions of different gross appearance are taken and immediately frozen at -15°C. In patients who come to postmortem examination, gross radioautographs may also be made, in which case the brain is removed, frozen rapidly, and sectioned either coronally or horizontally.

Autography of microscopic sections. Since conventional histologic fixatives were found to leach an appreciable fraction of the P^{32} activity from the tissue and to produce major shifts of the isotope within the tissue, all autographs have been made using unfixed quick-frozen material. Tissue sections are cut in a cold room at -15°C using a modification of the Linderstrøm-Lang technic (4,5). The sections for autography are cut at 20μ since, with the dosage levels permissible in humans, thinner sections often contain insufficient P^{32} to give an adequate image. These sections are allowed to melt on gelatin coated slides where they dry with little or no distortion. In the dark room a

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strip of Eastman No-screen x-ray film 2.5 x 7.5 cm is pressed firmly against the tissue section with a second glass slide and the entire 'sandwich' is sealed tightly with cellulose tape. The completed preparation is wrapped in opaque paper and stored in the cold room for the duration of the exposure, which is determined by the method of Steinberg and Solomon(6). Sections immediately preceding and following each 20μ section are cut at 8μ, fixed in formalin, and stained with hematoxylin-eosin for histologic correlation. When available, a sample of normal brain from each operation is mounted on the same slide with the tumor, providing a control which will necessarily receive exposure and development identical to those of the tumor section. Activity of tumor tissue can then be expressed relative to that of normal brain (1) without the necessity for further correction factors. At the conclusion of the calculated exposure, usually 7 to 20 days, the films are removed in the dark room and developed under controlled conditions. Each film is processed for 5 minutes in 50 cc of fresh Kodak D19 developer in a water bath at 20° $\pm$ 0.3°C. It is then stopped with 1% acetic acid and fixed and hardened in Kodak F5. Rigorous standardization of development is essential if it is desired to attempt quantitative comparison of autographs from different cases(6).

Autography of gross specimens. The beta particles of P^{32} (E_{max} 1.7 Mev) penetrate soft tissues (assuming density 1.0) to a maximum depth of 7 mm(7). This degree of penetration makes it possible to employ thick "slabs" of tissue (up to 7 mm) in order to obtain a survey autograph in minimum time. A large specimen may thus be studied within a period of 72 hours, for gross regions of high and low P^{32} uptake. Such autographs provide resolution which, although coarse, suffices to indicate areas of special interest for further study. The more laborious technic of microscopic radioautography may then be concentrated upon such areas. Since only 15% of the initial activity of the tissue decays in the first 72 hours, exposure time for the microscopic radioautographs is not unduly prolonged by this preliminary survey. The thick frozen section of brain obtained at operation or autopsy, including tumor, is kept constantly in a cold room at -10 to -15°C. Surfaces are trimmed so that irregularities will not impair the resolution of the autograph. Mean surface radioactivity is then estimated by means of a portable Geiger-Mueller counter and exposure time estimated (6). The specimen is covered with a single layer of aluminum foil (2.3 mg/cm^2) in order to protect the photographic emulsion; Eastman No-screen x-ray film is then applied under Wratten 6B light to each flat surface.§ The tissue and films are placed between cardboards, wrapped in opaque paper and stored in the cold room for the duration of the exposure. Films are routinely processed as described for microscopic radioautography. The frozen tissue specimen is photographed to provide a permanent record of its gross morphology.

Densitometry. Comparison of P^{32} uptake in various areas of a microscopic tissue section requires precise matching of corresponding areas in the radioautograph. In heterogeneous tumors it is desirable to measure densities in areas as small as 100μ in diameter. At the present time matching is accomplished by simple superposition, taking advantage of the configuration of the outlines of the tissue and the autograph. While this method is reliable in regions which can be readily identified, especially at the periphery of the section, it is difficult to be certain of the matching in central regions. A system employing simultaneous macroprojection of tissue and autograph for precise point-to-point correlation is now being developed. The radioautograph to be studied is projected on a ground glass screen through a microscope at suitable magnification. The machined aperture of the search unit of a Photovolt 500A

6. Steinberg, D., and Solomon, A. K., *Rev. Scient. Instr.*, 1949, v20, 655.

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§ Three sheets of film may be applied to each surface. The additional autographs so obtained, although inferior in resolution, assist in the recognition of any artefacts in the primary autographs.

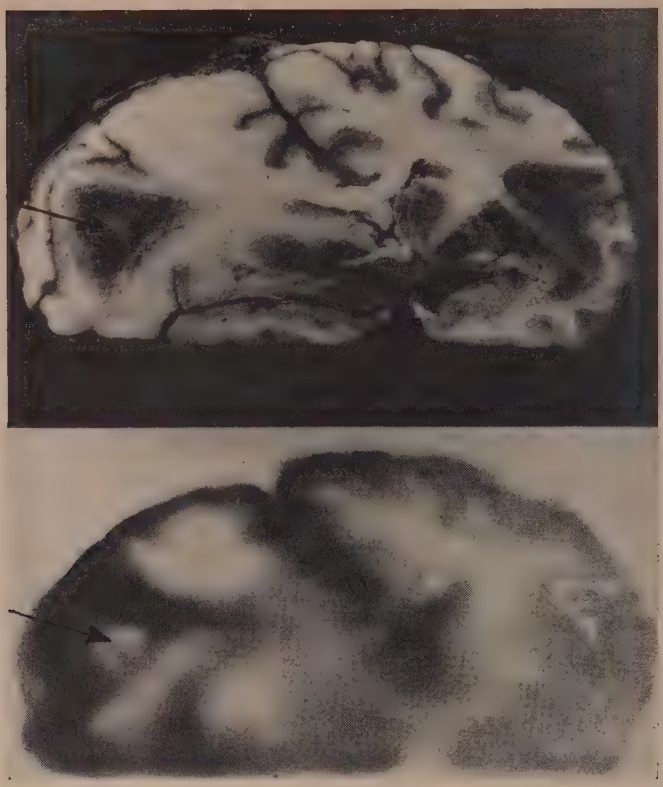
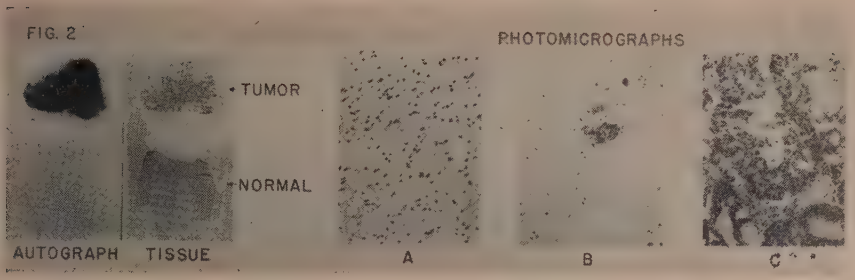


FIG. 1.
Gross radioautograph and photograph of brain slice containing glioblastoma multiforme. Note that the gray matter shows greater activity than the white but that the areas containing tumor are much denser than the gray. Note also that the necrotic region near the center of the tumor (see arrows) is much less dense than the surrounding tumor.

photometer is applied to the ground glass over the area whose density is to be measured. A standard Ansco optical wedge, projected in the same manner, is used for calibration between each set of readings. The light source, microscope and projection system are maintained in rigid alignment throughout the measurements. From the densities of the radioautographs relative P^{32} uptake can be calculated within $\pm 10\%$ for individual cases(6). Comparison from case to case requires standardization of dose and of time interval between P^{32} injection and operation, which is often made impossible by the clinical needs of the patient. Expressing uptake relative to that of the normal tissue makes the data more nearly comparable with respect to dose.

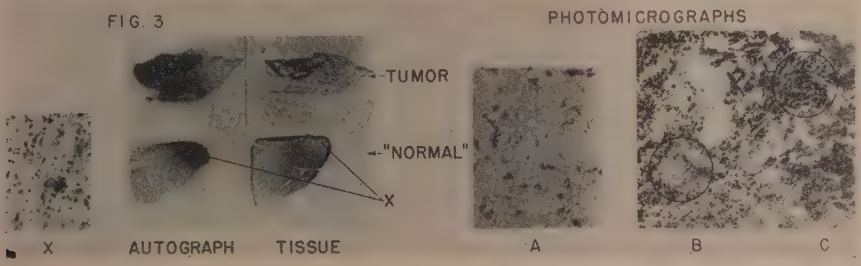
Discussion. Radioautographs of 18 cerebral tumors of various histologic types have been made employing these technics. Fig. 1 illustrates a gross radioautograph together with a photograph of the specimen from which it was made. Areas of interest in the gross autograph are removed from the frozen tissue slab in the cold room for microscopic autography. Fig. 2 illustrates the correlation of histologic appearance with microdensitometric determinations of autographic blackening in a bronchogenic carcinoma metastatic to the temporal lobe. Fig. 3 illustrates similar findings in a glioblastoma multiforme.



	Absolute density	Relative P^{32} content
A. Cerebral white matter near tumor	.07	1.0
B. Cerebral tissue at edge of tumor, containing a few tumor cells	.31	3.5
C. Actively growing region of tumor	.79	8.3

FIG. 2.

Bronchogenic carcinoma, metastatic to right temporal lobe. Patient received 3.9 mc of P^{32} 69.3 hours before operation. Exposure 11 days. Densities were measured in the regions of the radioautograph corresponding to photomicrographic areas A, B, and C.



	Absolute density	Relative P^{32} content
A. Cerebral gray matter with some glial reaction	0.07	1.0
B. Necrotic area in tumor	0.17	1.9
C. Nest of tumor cells	0.65	6.8

X—Tumor infiltration found in presumed normal control.

FIG. 3.

Glioblastoma multiforme, right temporal lobe. Patient received 3.6 mc of P^{32} 24.7 hours before operation. Exposure 26 days. Densities were measured in the regions of the radioautograph corresponding to photomicrographic areas A, B, and C.

The resolution of the autographs is adequate to permit microdensitometric measurements of adjacent areas as shown in the illustration. The radioautographic densities measured in this study reflect the total P^{32} uptake per unit volume of tissue. This uptake is a function both of the total content of phosphorus and of the rate of phosphorus replacement. Chemical analysis of nine brain tumors, including five different histologic types, showed significantly lower total phosphorus content in each case than in its control of normal brain (8). It would therefore appear reasonable to interpret increases in autographic density produced by brain tumor tissue as a reflection of increased metabolic activity.

Summary. Radioautographs of human brain tumors and of normal brain, employing P^{32}, have been made in 18 cases. A frozen section technic, without fixation, has been found necessary in order to prevent redistribution of the isotope within the tissues. Autographic blackening has been consistently much higher in tumor tissue than in brain,

8. Selverstone, B., and Moulton, M., personal communication.

but is subject to considerable variation within certain tumors. These variations, reflecting differences in uptake of radioactive phosphate ion by various portions of the tumor, may be correlated with certain histologic features in the stained sections. Quantitative microdensitometric measurements have been made, and relative P^{32} uptake determined from these data. An attempt is being made to correlate radioautographic features, especially in the gliomas, with histologic appearance and with prognosis.

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Estrogenic and Gonadotrophic Hormone Inhibiting Activity of Some Adrenal Cortical Substances.* (17886)

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The secretion of estrogens by the adrenal cortex has been observed clinically(1-3), and steroids possessing female sex hormone activity have been isolated from adrenal cortical extracts. Englehart(4,5) and Shirrmeister (6) obtained uterine growth with cortical extracts, and Beall(7) isolated estrone from ox adrenals. Desoxycorticosterone acetate has also been found to have estrogenic activity. Hoffman(8) and Gallardo(9) report uterine growth, and van Heuverswyn *et al.* (10) found mammary duct growth with this substance. In view of the administration of commercial adrenal cortical extracts (Lipo Adrenal Cortex, Adrenal Cortex Extract), desoxycorticosterone acetate (DCA), and 11-Dehydro-17-hydroxycorticosterone - 21 - acetate (Merck and Co., Inc.) in large amounts in experimental procedures, it is of practical importance to determine the doses of these substances which cause inhibition of the pituitary gonadotrophic hormone secretion and which result in uterine stimulation.

An ovariectomized immature female rat united in parabiosis with an intact littermate provides an assay technic by which a compound may be tested for both its activity in inhibiting pituitary gonadotrophic hormone secretion and in causing uterine growth. An estrogenic substance administered in an adequate dose to the ovariectomized rat prevents the post-castration hypersecretion of the gonadotrophic hormone and the resultant ovarian hypertrophy in the co-parabiont. The uterine hypertrophy of the castrate rat serves as an index of the estrogenic activity of the injected substance.

Procedures. Thirty-day-old female littermate rats of the Sprague-Dawley strain weighing between 65 and 75 g were joined in parabiosis according to the method of Bunster and Meyer(11) except that metal skin clips were used instead of silk sutures in closing the skin incisions. The right partners were ovariectomized at the time of parabiosis. One group of parabionts was left uninjected

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

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10. van Heuverswyn, *et al.,* PROC. SOC. EXP. BIOL. AND MED., 1939, v41, 552.

11. Bunster, E., and Meyer, R. K., *Anat. Rec.,* 1933, v57, 339.

TABLE I.
The Uterine Stimulating and Gonadotrophic Hormone Inhibiting Activity of Alpha-estradiol, Desoxycorticosterone Acetate, Compound E, Lipo Adrenal Cortex, and Adrenal Cortex Extract.

Compound	Dose (per day)	No. of pairs	Ovaries* (mg)	Uterus† (mg)
None	—	23	160 (110-230)	52 (32-90)
Alpha-estradiol	.0032 gamma	2	146	47
	.0065 "	2	122	60
	.009 "	2	16	45
	.012 "	3	25	51
	.025 "	3	25	135
	.050 "	2	29	168
DCA	250 gamma	2	133	80
	425 "	5	35	67
	500 "	2	20	77
	1000 "	2	23	123
	2000 "	1	35	110
Compound E	500 gamma	2	250	70
	2000 "	2	195	53
Lipo adrenal cortex	0.5 rat unit (.0125 cc)‡	2	170	45
	1 " " (.025 cc)	3	22	65
	2 " " (.05 cc)	4	20	117
	4 " " (.10 cc)	2	23	175
Adrenal cortex extr.	0.625 rat unit (.25 cc)‡	1	125	43
	2 " " (.8 cc)	3	118	52
	4 " " (1.6 cc)	3	65	45

* Ovaries of the left, intact partner.
† Uterus of the right, ovariectomized, injected partner.
‡ Original volume of the extract.

to serve as controls. In the remaining pairs, DCA, Lipo Adrenal Cortex, Adrenal Cortex Extracts, Compound E, and alpha-estradiol were assayed by subcutaneous administration to the ovariectomized partner daily for 10 days, beginning with the day of operation. DCA, Lipo Adrenal Cortex, and alpha-estradiol were dissolved in corn oil, and Compound E suspended in normal saline, so that a volume of 0.1 cc was injected daily. The Adrenal Cortex Extract was injected in relation to rat units without adjusting for volume. The animals were killed on the 11th day after parabiosis, and uterine and ovarian weights were determined.

Results and discussion. The results of these experiments are found in Table I. Ovarian weights of less than 100 mg are considered to indicate that partial inhibition of gonadotrophic hormone occurred, and weights of less than 50 mg are evidence that the inhibition was almost complete. Uterine weights of more than 100 mg indicate definite uterine stimulation by the injected substance.

It is seen that alpha-estradiol inhibited the pituitary secretion of gonadotrophic hormone when administered at a dose less than that required for uterine stimulation. This relationship was found to be true for several other estrogens tested in parabionts and in single *immature* rats, and will be reported in detail elsewhere(12). The DCA followed the same pattern, inhibiting the pituitary gonadotrophic hormone secretion at 425 γ per day, and producing uterine growth at 1 mg per day. On this basis, the gonadotrophin inhibiting activity of 425 γ of DCA is equivalent to approximately 0.01 γ of alpha-estradiol.

Compound E failed to inhibit the gonadotrophin at 500 γ or at 2 mg per day. Since the 2 mg dose caused a marked emaciation in

12. Byrnes, W. W., and Meyer, R. K., unpublished data.

the injected animals, higher doses were not administered.

Lipo Adrenal Cortex was found to inhibit the gonadotrophic hormone secretion at 1 rat unit per day and to produce uterine stimulation at 2 rat units per day. The gonadotrophin inhibiting activity of 1 rat unit of Lipo Adrenal Cortex can be estimated as equivalent to approximately 0.01 γ of alpha-estradiol. Adrenal Cortex Extract produced partial inhibition of gonadotrophin secretion in daily doses of 4 rat units. Due to the large amount of fluid required, higher doses were not tried. We can conclude that Adrenal Cortex Extract has less than one-fourth the gonadotrophin inhibiting activity of the Lipo Adrenal Cortex. The gonadotrophic inhibiting activity of the Lipo Adrenal Cortex and the Adrenal Cortex Extract is not proportional to their adrenal cortical activity, and is probably due to estrogenic substances obtained from the adrenals in the extraction procedures. To the best of our knowledge, DCA has not been isolated from these adrenal preparations, and does not account for the gonadotrophin inhibiting activity of the Lipo Adrenal Cortex or the Adrenal Cortex Extract.

The possibility of interference with the pituitary-ovarian interrelationship should be considered in the administration of these hormones in experimental and therapeutic procedures.

Summary. Lipo Adrenal Cortex and desoxycorticosterone acetate were found to stimulate uterine growth and to inhibit pituitary gonadotrophic hormone secretion when administered to female rats in relatively small amounts. Adrenal Cortex Extract partially inhibited gonadotrophin secretion but was less than one fourth as effective as the Lipo Adrenal Cortex. Large doses of Compound E failed to inhibit the gonadotrophin secretion. The secretion of gonadotrophic hormone is inhibited in *immature rats* by steroids in amounts less than those which result in uterine growth.

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Effect of Folic Acid, Aminopterin and Vitamin K on Growth of Roots of *Allium cepa.** (17887)

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Previous reports have described the effect of colchicine on nuclear divisions in onion root tips(1) and in human tumors(2). The action of colchicine, acenaphthene and a combination of colchicine and x-ray treatments have also been reported(3). It seemed of interest to study the effect of folic acid (pteroylglutamic acid) and its antagonist aminopterin (4-amino pteroylglutamic acid) to determine the influence these agents have on the rate of growth of fundamental tissue since they have been used as theraputic agents in the treatment of cancer. Vitamin K substitute (2-methyl 1,4-naphtho-hydroquinone-diphosphoric acid ester, tetra sodium salt plus 6 H_2O) another agent that gained some popularity in the treatment of neoplasia was studied and compared with colchicine, folic acid and its antagonist in the light of recent claims that this substance combined with

* Supported in part by a grant-in-aid from the Damon Runyon Memorial Fund.

1. Levine, M., and Gelber, S., *Bull. Torrey Bot. Club*, 1943, v70, 175.

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3. Levine, M., *Bull. Torrey Bot. Club*, 1945-1946, v72, 563; v73, 34; v73, 167.

TABLE I.

Agent and conc., g/ml	Action on root elongation: No. of roots	Avg total growth 10 days, mm	% inhibition*	Avg mitotic frequency per 2000 cells: 1st 5 days	2nd 5 days	% reduction†: 1st 5 days	2nd 5 days
Controls	80	84.3	00.0	2.62	3.46	0.0	0.0
F.A. 10^{-5}	32	10.3	87.8	1.76	1.06	32.8	69.4
" 10^{-7}	56	31.5	62.6	2.54	1.52	3.1	56.1
" 10^{-9}	32	44.3	47.4	1.86	1.56	29.0	54.9
Amin. 10^{-7}	32	2.5	97.0	0.23	1.32	91.3	61.8
" 10^{-9}	32	38.7	54.1	2.16	1.58	17.6	54.3
" 10^{-10}	32	52.2	38.1	1.98	1.18	24.4	65.9
Vit. K subs.							
10^{-4}	32	6.2	92.6	1.56	2.06	40.5	40.5
10^{-5}	32	31.5	62.6	2.18	1.40	16.8	59.5
10^{-7}	32	52.8	37.4	2.34	1.54	10.7	55.5

* The % inhibition is the difference of the % of total growth from 100%. The % total growth is the fraction the treated roots grew as compared to the controls.

$$\% \text{ inhibition} = 100\% - \frac{\text{total growth of treated roots}}{\text{total growth of controls}} \times 100$$

† The % reduction is the difference of the % of mitosis in the treated roots from the controls divided by the % of mitosis in the controls.

$$\% \text{ reduction} = \frac{\% \text{ mitosis controls} - \% \text{ mitosis treated roots}}{\% \text{ mitosis controls}} \times 100$$

x-rays inhibited growth of tissue components grown *in vitro*(4).

Methods and materials. *Allium cepa* var. Yellow Globe, the root tissue of which is readily adapted to the root smear technic, was used in this study. The bulbs were selected on the basis of approximate uniformity of weight (60 to 80 g) freedom from bruises and infections. The onions were germinated in tap water in opaque cylindrical jars of 240 ml capacity for 3 days. Each experimental series was started with a larger number of bulbs than was actually treated, more than 200 onions were used. After the germination period, the selected onions were placed in water in which the agent under investigation was dissolved or suspended. Folic acid and aminopterin solutions were made from stock suspensions containing 20 mg/100 ml. Attempts to dissolve these materials by the addition of small quantities (1 to 5 ml) of 95% alcohol or ether failed. The vitamin K substitute (Hoffmann-LaRoche, Synkovite) was water soluble and the solutions were made from stock containing 100 mg/100 ml. Each of these substances was tested at an initial concentration of 10^{-7} g/ml. Linear growth of the root was measured at the time of immersion and thereafter daily for 10 days. Root tips removed for cytological examination were prepared by the root smear technic and stained with Sudan Black B as described by Cohen(5) with the omission of the propionic acid.

Observations. Folic acid at concentrations of 10^{-5}, 10^{-7} and 10^{-9} g/ml, aminopterin at concentrations of 10^{-7}, 10^{-9} and 10^{-10} g/ml, and vitamin K substitute at concentrations of 10^{-4}, 10^{-5} and 10^{-7} g/ml had an inhibitory effect upon the linear growth of onion roots. Folic Acid at the above concentrations had no other obvious toxic effect upon the roots. These roots were glistening white, turgid and appeared normal. At concentrations of 10^{-7} and 10^{-9} g/ml the increment in length after 1 day immersion was close to that of the controls, but thereafter the difference between the two became more pronounced. Folic acid at a concentration of 10^{-5} g/ml suppressed growth during the whole immersion period. The average total lengths of the treated roots and controls appear in Table I and Fig. 1. Aminopterin at a concentration of

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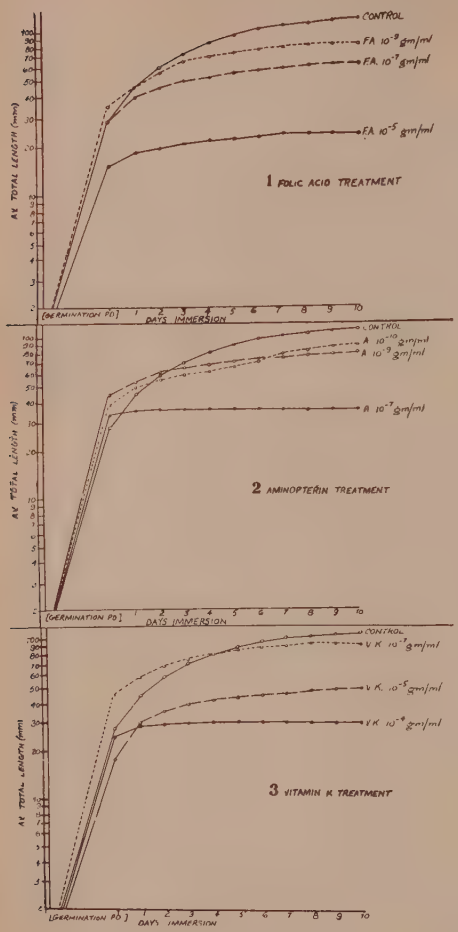

FIG. 1.

10^{-7} g/ml caused some of the roots to become flaccid and slightly yellowish in color after 5 days immersion. Throughout the experimental period aminopterin at this concentration depressed root growth and after 4 to 6 days immersion, root growth ceased. With concentrations of 10^{-9} and 10^{-10} g/ml the average daily increments in length were smaller than those of the controls. The total lengths of the treated roots and controls are shown in Fig. 2. Vit. K substitute at concentrations of 10^{-4}, 10^{-5} and 10^{-7} g/ml showed inhibitory effects on root growth. At a concentration of 10^{-4} g/ml the agent showed an effect similar to aminopterin at a concentration of 10^{-7} g/ml. After immersion of 5 to 6 days root growth ceased and some roots became flaccid and yellowish in color. Vit. K substitute at concentrations of 10^{-5} and 10^{-7} g/ml showed inhibitory effects of the same order of magnitude as folic acid at concentrations of 10^{-7} and 10^{-9} g/ml (Table I and Fig. 3). These studies showed that in every instance growth inhibition increased with the concentration of the agent (Table I and Fig. 1).

Microscopic studies of the treated and control roots were made by the smear technic together with fixed and paraffin sectioned material. The tissue was examined for karyokinetic aberrations and the cells counted under high dry magnification. The percentage of mitoses was based on counts of 2000 or more cells. In all but 2 instances there is a fall in the 5-day averages of the mitotic index as the immersion time lengthens (Table I). The variations in the percent of mitosis in the treated roots may represent inherent properties of the tissue as the controls manifest similar variations. The number of mitoses in the controls is consistent with the counts made on similar material by Levine and Gelber(1). No cellular abnormalities such as arrested metaphases or polyploidy were noticed in the treated roots. In general, the treated roots showed a smaller percent of mitosis than the controls. While the percent of division figures was depressed there were mitotic stages present in all of the roots after treatment. The inhibition of growth by these agents may be due to an effect upon the ability of the root to elongate and with some impairment of the mitotic process.

Discussion. In the studies described here folic acid at a concentration of 10^{-5} g/ml and aminopterin at a concentration of 10^{-7} g/ml had similar inhibitory effects on growth (Table I). The ratio of these concentrations, folic acid to aminopterin is 100:1, the same relative potencies observed when these compounds were used on frog oviducts(6). Mitchell and Simon-Reusse(4) studied the effect of vitamin K substitute in combination

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with x-rays on tissue cultures of chick fibroblasts. Vit. K substitute alone and in combination with x-rays, it was observed, produced mitotic inhibition. In general in our studies it was observed that the treated roots besides showing an inhibition in linear growth had a lower mitotic percentage than the controls though it must be emphasized that no karyokinetic abnormalities were observed.

Summary. The effect of folic acid, aminopterin and vitamin K substitute dissolved or suspended at given concentrations in water, on the growth and development of roots from bulbs of *Allium cepa* was investigated. Folic acid at concentrations of 10^{-5}, 10^{-7} and 10^{-9} g/ml, aminopterin at concentrations of 10^{-7}, 10^{-9} and 10^{-10} g/ml and vitamin K substitute at concentrations of 10^{-4}, 10^{-5} and 10^{-7} g/ml inhibited the linear growth of roots of *Allium cepa*. The percent of mitosis in the treated roots was in general lower than that of the controls. No abnormalities of the mitotic figures in the treated root tips were observed. The inhibition of growth by these agents may be due partially to an interference with the process of cellular elongation and differentiation following karyokinesis and partially by reducing the rate of nuclear division.

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Biuret Reaction as Applied to Determination of Antibody Nitrogen. (17888)

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(Introduced by H. Molitor)
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The quantitative biuret reaction(1) has been successfully applied to the determination of protein in urine(2), blood serum(3) and cerebrospinal fluid(4). Since the method is simple and rapid, it appeared desirable to investigate its use in immunochemical procedures. Various proteins may yield biuret colors whose absorption maxima differ slightly in intensity and position(5). In a series of determinations of optical density per mg N at 540 mμ relative to bovine serum albumin, we obtain values of 0.95, 0.98, 1.08, and 1.12, respectively for horse, rabbit, rat and dog serum, 1.01 for crystalline egg albumin, and 0.98 for bovine γ-globulin. With antigen-antibody precipitates in which the components give similar absorption maxima, the error encountered should be negligible. Correlation with nitrogen content may be obtained by the use of proper standardization procedures.

Materials and methods. Antigens. Crystalline egg albumin and crystalline bovine albumin were used in these studies. For immunization procedures, the albumins were precipitated with alum; for antibody precipitation, they were dissolved in 0.85% NaCl solution. The nitrogen contents were determined by the micro-Kjeldahl method. *Antisera**. Albino rabbits weighing about 1.5 kg were given 4 series of 4 intravenous injections of antigen increasing from 1.5 mg to 7.5 mg. Rest periods of 3 days were allowed between series, and 7 days after the last injection blood was withdrawn aseptically. Sera were preserved by the addition of 0.01% sodium merthiolate. *Precipitation of antibody.* Antibody was precipitated according to the method of Heidelberger and Kendall(6) as described by Kabat and Mayer(7). *Biuret reaction.* The biuret reagent described by

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* The antisera were prepared by Miss E. Jane Tullius.

Kingsley(8), was prepared by mixing 400 ml of a 1% aqueous solution of copper sulfate pentahydrate with 2000 ml of a 1.4% aqueous solution of sodium hydroxide. Analytical reagent grade chemicals were used. The reagent is stable for several months when protected from light.

To the antigen-antibody precipitates were added 1 ml of water followed by 6 ml of biuret reagent. The solutions were then transferred to ½ inch square cuvettes and their optical densities (–log T) were compared with that of a water-reagent blank at 540 mu. A Coleman Universal Spectrophotometer, Model 11 was used. A dried sample of crystalline bovine albumin† (fraction V, Armour) was employed as a reference standard. With the biuret reagent, this protein yielded solutions with optical densities of 0.302 per mg N. Precipitates from rabbit anti-bovine albumin serum plus homologous antigen, containing 0.2 to 0.7 mg N (micro-Kjeldahl), yielded average optical density values of 0.309 per mg N. In a series of measurements with similar precipitates, the optical density per mg N for the bovine albumin was multiplied by the ratio 0.309/0.302 to obtain optical density per mg N for the precipitates.

Kjeldahl method. A micro procedure with selenium(9) as the digestion catalyst was used. This catalyst may yield unreliable results(10), but in the present work nitrogen values obtained with the selenium catalyst were similar to those obtained with a copper catalyst.

Results. Data on the antibody N content of 7 rabbit antisera, as determined by the biuret and Kjeldahl methods, are given in Table I; one was an anti-bovine albumin serum and the 6 others were anti-egg albumin sera. With 3 of the 7 sera the 2 methods yielded values differing by 0.02 mg antibody N per ml of serum, while differences of 0.01 mg or less were obtained with the 4 remaining sera. Thus analysis of duplicate precipitates by the two methods agreed within ± 0.02 mg for samples containing 0.1 to 0.5 mg of antibody N.

TABLE I.
Comparison of Antibody Nitrogen Determinations with the Biuret and Kjeldahl Methods.

Rabbit antiserum No.	Antibody N, mg/ml serum	
	Biuret	Kjeldahl
Anti-bovine albumin 1	.46	.46
	.48	.47
" " " 1	.45	.45
(10% less antigen in antigen-antibody mixture)	.45	.45
Anti-egg albumin 1	.50	.52
	.51	.51
	.51	.52
" " " 2	.27	.27
	.26	.26
" " " 3	.31	.29
	*	.29
" " " 4	.23	.24
	.22	*
" " " 5	.26	.28
	.29	.26
" " " 6	.10	.12
	.11	*

* Tube broken during centrifugation.

Summary and conclusions. A quantitative biuret method for the determination of protein has been applied to the measurement of antibody N. The nitrogen contents of duplicate antigen-antibody precipitates from rabbit anti-bovine albumin serum and rabbit anti-egg albumin sera, plus the homologous antigens, were determined by the biuret and micro-Kjeldahl methods. The results agreed within ± 0.02 mg nitrogen with precipitates containing 0.1 to 0.5 mg antibody nitrogen.

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Effect of Intravenous Injection of Tuberculin on Leucocytes of Normal and Tuberculous Rabbits.* (17889)

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The specific cytotoxic effect of tuberculin for certain cells of the tuberculous animal, first shown by Rich and Lewis(1), has been confirmed by others(2-4) using tissue cultures. Recently Favour and his group(5,6) have reported that in the presence of tuberculin, leucocytes of tuberculous animals and humans are lysed *in vitro*. It now appears that this phenomenon is referable to a plasma factor, since leucocytes from normal as well as tuberculous humans react in the same fashion when suspended in plasma from tuberculous individuals(7). Similar phenomena occur *in vivo*(8,9). Furthermore, the significance of changes in leucocytic formulae in tuberculosis has long been recognized by clinicians. Cunningham and Sabin(10), Sabin and Doan(11), and Wiseman and Doan(12) focused attention on changes in the monocyte-lymphocyte ratio, and showed that an increase in lymphocytes is associated with the healing stage of active tuberculosis, while a depression in relative numbers of lymphocytes denotes progression of the disease.

This peculiar role of the lymphocyte, and its importance in the passive transfer of tuberculin sensitivity(13) has prompted as to study the effect of tuberculin *in vivo* on the lymphocytes of tuberculous animals.

Experimental. Male albino rabbits were inoculated subcutaneously with the Ravenel strain of *Mycobacterium tuberculosis* var. *bovis* propagated on Petragnani's medium. At the time of use, about two months after infection, each rabbit had begun to lose weight and showed a definite cutaneous sensitivity to tuberculin. These rabbits and normal rabbits were given 0.25 or 0.50 mg P.P.D. (Purified Protein Derivative) intravenously. Differential and total leucocyte counts were made from a free-flowing drop of blood from the marginal ear vein. Certified pipettes were used in diluting the blood for the total leucocyte count and both chambers of the Neubauer Brightline Counting Chamber were counted. The smears were stained immediately with Field's stain and 200 cells in various parts of the slide were counted. Counts were made at appropriate intervals during the day preceding the injection of P.P.D. to control the effect of the normal daily fluctuation in total leucocyte and in differential white cell numbers which is accentuated in tuberculosis.

Results. Inspection of the table indicates a leucocytopenia due to the sharp drop in the number of circulating lymphocytes. The studies show that this reaction to tuberculin is progressive because, while the number of circulating lymphocytes had begun to decrease in 3 hours, the maximum decrease was not reached until 6 to 9 hours after the intra-

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TABLE I.
Effect of P.P.D. on Hematological Picture of Normal and Tuberculous Rabbits.

Group	Hr after tuberculin	WBC* ×100	Neutrophiles ×100	Lymphocytes ×100	% Neutrophiles	% Lymphocytes	% Monocytes
I	0	110	24.	80.5	24	75	1
	3	84	39.4	44.5	47	53	0
	6	46	27.6	18.4	60	40	0
	9	70	43.5	26.6	62	38	0
	13	116	61.5	55.5	53	47	0
	28	116	25.5	86.6	22	74	4
II	0	154	57.	78.5	37	51	12
	6	48	31.2	16.3	65	34	1
	9	70	45.	25.2	64	36	0
	24	186	82.	78.	44	42	14
III	0	252	116.	93.	46	37	17
	6	158	120.	23.8	76	15	9
	12	250	160.	45.	64	18	17
	24	206	82.5	101.	40	49	11
IV	0	92	30.	61.	33	67	0
		118	46.	72.	39	61	0
	6	122	39.	83.	32	68	0
		134	61.5	72.4	46	54	0
	9	120	28.	89.	24	74	2
		114	42.1	71.9	37	63	0
	13	100	29.	71.	29	71	0
		118	43.6	73.	37	62	1
	26	108	35.6	72.5	33	67	0
		120	39.6	79.1	33	66	1

Group I. Four tuberculous rabbits with 2 mo. infection. .5 mg P.P.D.
Group II. Four tuberculous rabbits with 2 mo. infection. .5 mg P.P.D.
Group III. Four tuberculous rabbits with 3 mo. infection. .25 mg P.P.D.
Group IV. Two normal rabbits.
* Avg count of all the animals in each group.

venous injection of P.P.D. Between 75-80% of the lymphocytes disappear from the circulating blood during this period, after which they increase slowly and reach or may exceed their previous level in 24 hours. That the leucocytopenia is due entirely to the disappearance of the lymphocytes is indicated by the fact that the number of circulating neutrophiles shows little decrease. In fact, there is a sharp rise in the total number of neutrophiles between 9 and 12 hours after P.P.D. is injected. Such changes do not occur in normal animals given the same treatment.

Discussion. Favour(5) found that lysis by tuberculin of both lymphocytes and neutrophiles occurs *in vitro,* while our results indicate that little or no observable destruction of neutrophiles occurs. However, since a dynamic state exists *in vivo* that is not duplicated *in vitro,* a rapid production of neutrophiles under these conditions may mask their actual destruction. Moreover, analysis of Favour's data suggests that although both cell types are lysed *in vitro,* the lymphocytes are destroyed at a greater rate than are the neutrophiles.

In active tuberculosis, a certain amount of autoinoculation with tuberculo-protein probably occurs continuously, and it is possible that this is responsible for the relative lymphopenia characteristic of progressive tuberculosis.

Summary. Following the intravenous injection of P.P.D. into tuberculous rabbits there is a decrease in the total number of white cells and lymphocytes. The maximum decrease occurs in 6 to 9 hours and is followed by a gradual return to normal. There is also frequently a delayed increase in neutrophiles.

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Measurement of Renal Hemodynamics in Man by the "Slope Method" without Urinalysis. (17890)

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Since the introduction by Homer Smith of methods for measuring renal plasma flow and glomerular filtration rate, attempts have been made by several investigators to adapt his procedures to clinical use. The usual method, as developed by Smith, involves accurately timed collections of urine, the maintenance of an intravenous infusion at a constant rate, the collection of frequent blood samples, and the analyses of both plasma and urine. The technic has not been generally applicable clinically largely because of its intricacies. Landowne(1), and Alving and Miller(2) attempted to simplify the procedure for measuring inulin or mannitol clearance, but simultaneous collections of both blood and urine specimens were still necessary. A method has been described by Earle and Berliner(3) and by Berger, Farber and Earle(4) that involves collections of blood specimens only. Nevertheless, as a preliminary step, one must determine the rate of infusion of mannitol or PAH necessary to maintain a constant blood level. Beyer(5) has successfully measured renal plasma flow by determining the clearance of sodium para-aminohippurate (PAH) following oral administration of this compound, thus obviating the necessity for maintaining a constant infusion. Newman(6) suggested a principle for determining mannitol clearance without the collection of urine. A substance is administered intravenously by a single injection. The proportion of that substance disappearing from the plasma per unit of time represents the fraction excreted by the kidney per unit of time, assuming no extrarenal loss. The product of that proportion (S) and the volume of fluid in which the total substance is distributed (Vc) is equivalent to the volume of fluid that contains the amount removed by the kidney per unit of time or, by definition, the renal clearance (C). Thus, $C = VcS$. Preliminary results from our application of this principle to measure clearances of mannitol and PAH in animals(7) and in man(8) were previously reported. The present communication consists of our expanded results and conclusions from 17 experiments on 16 patients.

Method. Patients were fasted overnight and well hydrated before the experiments. An indwelling catheter was inserted into the bladder and urine and blood blanks were collected. Precisely measured amounts of PAH (usually 1,380 mg) and mannitol* (usually 12,000 mg) were administered intravenously from calibrated syringes, and 50 minutes were allowed for equilibration of these substances in their fluid compartments. The bladder was emptied, and five blood samples were taken from the antecubital vein at 10 minute intervals. Two successive 20 minute urine collection periods were run. The bladder was irrigated with 20 cc saline and flushed with air for each collection. In 5 experiments, arterial blood samples were also drawn through an indwelling needle in the brachial artery. Plasma and urine specimens were analyzed for PAH and mannitol. Before colorimetric analysis, plasma filtrates

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TABLE I.
Comparison of Slope Clearance with Urine Clearance of PAH and Mannitol Based on Analysis of Venous Plasma.

Subject		Mannitol			PAH				
		C_u	C_s	C_s/C_u	C_u	C_{s_m}	$C_{s_{pah}}$	C_{s_m}/C_u	$C_{s_{pah}}/C_u$
1.	Eczema	96.8	98.4	1.02	431	441	682	1.02	1.58
2.	Arthritis	72.0	103.7	1.44	244	236	301	.97	1.23
3.	No disease	77.7	80.9	1.04	334	333	544	1.00	1.63
4.	Hypertension	88.3	79.0	.90	363	236	418	.65	1.15
5.	Rheum. fever	56.8	85.5	1.51	249	254	549	1.02	2.20
6.	" "	76.7	79.2	1.03	363	393	339	1.08	.94
7.	" "	118.2	153.0	1.29	475	396	854	.83	1.80
8.	Arthritis	90.5	114.7	1.27	521	368	670	.71	1.29
9.	Lymphosarcoma	97.8	159.0	1.62	351	346	497	.99	1.42
10.	Pneumonia	102.0	121.5	1.19	576	420	671	.73	1.16
11.	Rheum. fever	150.0	172.0	1.15	579	552	1080	.95	1.87
12.	Malignancy	37.0	73.2	1.98	97	98	122	1.01	1.26
	Avg			1.29				.91	1.46

Symbols: C_u = urine clearance (cc/min.). C_s = slope clearance.
C_{s_m} = Slope clearance using volume of distribution of mannitol.
$C_{s_{pah}}$ = Slope clearance using volume of distribution of PAH.

and diluted urines were hydrolyzed with 0.4 N HCl for 3½ hours at 97° C to free the PAH acetylated by the liver. The plasma concentrations of PAH and mannitol in each case fell in a straight line when log of plasma concentration was plotted against time as a straight abscissa. This line, extrapolated back to the time of injection, indicated the theoretic plasma concentration of PAH and mannitol at this time. This value, divided into the total amount injected, gave the volume of distribution. Thus:

$$Vc = \frac{\text{mg injected}}{\text{P (mg/cc) at zero time}}.$$

Slope (S) was determined from the graph of the falling plasma concentrations of PAH and mannitol by the formula: $S = \frac{\ln (A—B)}{t}$

in which "A" and "B" are the plasma concentrations at two different points on the falling curve, "t" is the time interval separating those points, and "ln" is the natural logarithm. Slope clearance was determined from these data by the formula previously described, C = Vcs. For comparison, determination of simultaneous clearances by the urine collection method was made by satisfying the formula $C = \frac{UV}{P}$. P was determined 2 minutes before the mid-point of each urine collection period to allow for the lag between renal excretion and appearance of urine in the bladder. Clearances based on analysis of arterial and venous plasma were determined in 5 experiments both by the slope method and by the urine collection method. In 4 experiments, urine was collected for 24 hours and analyzed for mannitol and PAH to determine the recovery of these substances in the urine.

Results. Results are presented in Tables I, II, and III. The slope clearance of PAH determined by the volume of distribution of PAH was uniformly greater than simultaneously measured urine clearance. We considered, however, that there is justification for use of the volume of distribution of mannitol rather than of PAH in the calculation of slope clearance, since perhaps the volume of distribution of PAH cannot be accurately measured from the falling curve. Its rapid removal by the kidney from the plasma during the 50 minute mixing and equilibration period prevents plasma levels of PAH from reflecting correctly the level throughout its volume of distribution at any one time. The rapid fall of PAH concentration in the plasma gives a falsely high value for the volume of distribution of this substance. Therefore, using the volume of distribution of mannitol, we found close correlation between venous slope clearance and urine clearance in 8 of 17 ex-

TABLE II.
Comparison of PAH and Mannitol Slope Clearances of Arterial and Venous Plasmas and Correlation with Corresponding Urine Clearances.

	Mannitol						PAH						
	Arterial			Venous			Arterial			Venous			
	C_s	C_u	C_s/C_u	C_s	C_u	C_s/C_u	C_{u_v}/C_{a_a}	% recovery	C_s	C_u	C_s/C_u	C_s	C_u	C_s/C_u	C_{u_v}/C_{a_a}	% recovery
1.	172	154	1.12	186	135	1.38	.88	99	439	880	.50	478	622	.77	.71	86
2.	251	128	1.96	180	128	1.41	1.	90	474	908	.52	352	750	.47	.83	88
3.	169	129	1.31	169	129	1.31	1.	101	429	824	.52	429	824	.52	1.00	84
4.	189	191	0.99	132	134	0.99	.7	*	457	584	.79	318	485	.66	.83	*
5.	112	72	1.55	104	61	1.71	.85	98	308	445	.69	191	376	.51	.84	93
Avg			1.39			1.36	.88	97			.62			.59	.84	88

* Urine accidently discarded. Symbols: C_s—Slope clearance based on volume of distribution of mannitol. C_u—Urine clearance. Diagnosis: 1. Convalescent pneumonia; 2. Schizophrenia; 3. Possible tuberculosis; 4. Hyperthyroidism; 5. Plasma cell myeloma.

TABLE III.
Uncorrected Urine Clearances of PAH (in cc/min.) in 2 Successive 20 Minute Periods with Falling Plasma Concentrations.

Patient	C_1	C_2	C_2/C_1
J.	485	447	.92
L.	259	229	.88
M.L.	335	332	.99
G.	365	360	.99
M.P.	225	272	1.21
,,	360	366	1.02
W.	466	484	1.04
D.	490	552	1.13
G.N.	361	341	.94
C.	582	570	.98
J.A.	451	675	1.49
F.	622	675	1.08
M.M.	750	1054	1.41
V.	824	1069	1.30
E.	485	567	1.17
N.	376	492	1.31
Avg			1.12

periments. In these 8 experiments, the average quotient of slope clearance and urine clearance was 1.005. In the total experience with 17 determinations, however, the average quotient of slope clearance of PAH, based on the volume of distribution of mannitol, and urine clearance was 0.91 with a range of 0.47 to 1.08.

The correlation between slope clearance and urine clearance of mannitol was less satisfactory. The average quotient of the mannitol slope clearance and urine clearance was 1.47 with a range of 0.90 to 1.98.

Urine clearances of mannitol and PAH of venous blood, when plasma concentrations were falling, were usually smaller than simultaneous clearances of arterial blood (Table II). The average venous clearance of PAH was 84 percent of the arterial clearance, and the average venous clearance of mannitol was 88 percent of the arterial clearance.

Percentage recovery of injected PAH and mannitol is indicated in Table II. The average percentage recovery of PAH in 4 experiments was 88%, and of mannitol, 97%.

Recently, Newman(9) has presented data indicating that urine clearance of PAH is not independent of plasma concentration

9. Newman, E., Kattus, A., Genecin, A., Genest, J., Calkins, E., and Murphy, J., *Bull. Johns Hopkins Hosp.*, 1949, v84, 135.

when the plasma concentration is falling. He found that the clearance fell in successive periods as the plasma concentration of PAH decreased. Hence, we compared the urine clearances in two successive 20-minute periods as the plasma levels fell, following the 50-minute mixing and equilibration period. Results are shown in Table III. It appears that in our series, there was no consistent variation of C_{pah} with falling plasma concentration. The average ratio of the second period to the first was 1.12, indicating slightly greater clearances when the plasma level was lower.

Comment. The validity of slope clearance depends on the demonstration that no significant extra-renal loss of PAH and mannitol occurs. That such loss does not occur has been shown by Elkinton(10) and by Clark and Barker(11). To confirm this point further, we determined the plasma levels of PAH and mannitol following injections of these substances in 2 completely anuric patients with the lower nephron syndrome. After equilibration, plasma levels remained constant, indicating that with complete suppression of renal function there is no loss of PAH and mannitol by a extra-renal mechanism. Houck(12) has shown in dogs that the extra-renal clearance of PAH is 6% of the total urine clearance, and the extra-renal clearance of mannitol is 4% of the total urine clearance. Our results fail to show that mannitol clearance can be accurately determined by the slope method without urine collections. In most of our experiments, the slope clearance of mannitol was significantly lower than the simultaneously measured urine clearance. The reason for this discrepancy is not apparent. Slope clearance of PAH, based on the volume of distribution of mannitol, was more frequently confirmed by the simultaneously measured urine clearance, as shown in cases, 1, 2, 3, 5, 6, 9, 11 and 12, Table I. Although measurement of clearance by the slope method seems theoretically sound, important factors influence its accuracy. One is the possibility that plasma levels of PAH and mannitol are lower than their concentrations throughout their volumes of distribution because of their rapid removal from the plasma by the kidney. Furthermore, PAH and mannitol are likely never completely distributed because they are being excreted as they are distributed. As a result of both of these factors, the calculated volume of distribution may be too high, raising, in turn, the derived clearance value. Variation in the time between excretion of urine by the kidney and its appearance in the bladder may occur, perhaps depending upon the rate of urine flow. We arbitrarily determined the concentration of PAH 2 minutes before the midpoint of each urine collection period in calculating urine clearance. Variation in the lag more or less than 2 minutes would induce some error into the calculated clearance. Nevertheless, the close correlation between slope clearance and urine clearance observed in 8 experiments seems inexplicable by coincidence alone. The results in these instances lend encouragement to seek further the factors responsible for the lack of correlation in the remaining cases.

Our results are in accord with the experience of Brun(13) and Newman(9) that venous blood clearances of diodrast, PAH, inulin, and mannitol are lower than arterial clearances when the plasma levels of these substances are falling.

Summary. 1. We have determined PAH and mannitol clearances by the slope method without urine collection in 17 experiments, on 16 patients, comparing the results with urine clearances simultaneously measured.

2. Values of PAH clearance determined by the slope method and based on the volume of distribution either of PAH or mannitol, failed to be confirmed consistently by simultaneously measured urine clearances. PAH slope clearance agreed more often with urine clearance when the slope clearance was based on the volume of distribution of mannitol than when based on the volume of distribution of PAH. This agreement occurred too frequently to be fortuitous.

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13. Brun, C., Hilden, T., and Raaschon, F., *J. Clin. Invest.*, 1949, v28, 144.

3. Values for mannitol clearance determined by the slope method were consistently lower than simultaneously measured urine clearances; therefore, mannitol clearance cannot be determined by the slope method.

4. Clearance of venous plasma when plasma concentration is falling is lower than clearance of arterial plasma.

5. Neither mannitol nor PAH is metabolized, but they are excreted exclusively by the kidney, since a high urinary quantitative recovery of each was obtained in 24 hours, and neither disappeared from the plasma of two anuric patients.

6. Although the variation was not consistent, there was an average slightly greater urine clearance at lower plasma levels of PAH than at higher levels as the plasma concentration decreased.

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Pharmacology of Isomeric Thiocyanobenzoic Acids.* (17891)

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In a former communication(1) the authors and Tawab studied the pharmacologic responses to meta- and para-thiocyanobenzoic acids. In the main, the actions elicited by the two compounds were identical. Upon intravenous injection in the dog and rabbit they produced marked depressor responses. These were accompanied by electrocardiographic changes. The meta- and para-thiocyanobenzoic acids relaxed smooth muscle. In low concentrations they inactivated brain dehydrogenases and cytochrome oxidase. This effect was not shared by the thiocyanate ion. Through the kindness of Dr. G. F. D'Alelio† a sample of orthothiocyanobenzoic acid was provided. It is the most difficult of the three isomers to prepare. We decided to compare its pharmacologic activity with that of the meta and para compounds. Experimental studies were conducted with the soluble sodium salts of the 3 isomeric acids.

Blood pressure studies—dogs. The blood pressure studies were conducted on 6 dogs under ether anesthesia. Carotid artery blood pressure was recorded and injections of the various compounds were made into the saphenous vein. Solutions of 0.5% and 1.0% of the sodium salts were used. In our former studies it was demonstrated that the meta- and para-thiocyanobenzoic acids produced a marked depressor response when a dose of 0.5 cc/kg of a 0.5% solution was injected. The respiration was increased in rate and amplitude. This was followed by a depression of respiration. A constant finding was a marked accentuation of the T-wave of the E.C.G. in Lead II. A typical blood pressure tracing is shown in Fig. 1. The effect of the ortho compound illustrates that this isomer does not share with the meta and para compounds the capacity of eliciting a depressor response in non-toxic doses. Larger doses produce a progressive fall in blood pressure which is invariably fatal. In contrast to the constant E.C.G. finding of T-wave accentuation with the meta and para compounds, the ortho compound produced no changes in the E.C.G. in non-toxic doses. Toxic doses caused a depression of the Q R S complex and a flattening to an inversion of the T-wave.

Action on smooth muscle. The action of the meta- and para-thiocyanobenzoic acids on the smooth muscle preparations of the rabbit's intestine and the rat's uterus *in vitro* was markedly relaxant. A concentration of

* The expense of this investigation was defrayed in part by a grant from the Bressler Alumni Research Fund.

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† Director of Research, Koppers Company, Inc., Pittsburgh, Pa.

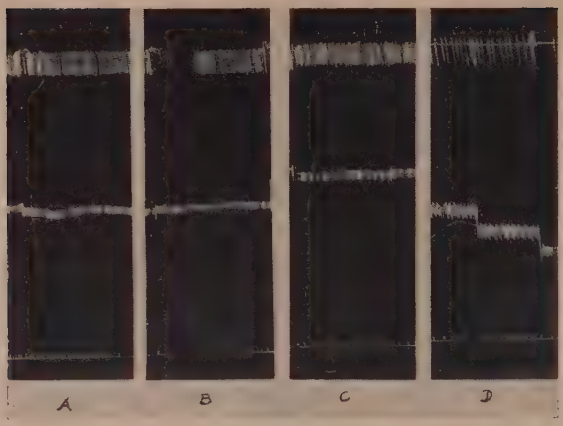

FIG. 1.

Effect of o-Thiocyanobenzoic acid on the blood pressure of the dog (male dog, 10.2 kg, ether anesthesia; normal blood pressure 114 mm Hg). A. Normal tracing. B. .5 cc/kg 1% sol. of o-thiocyanobenzoic acid. C. After 60 min. no appreciable change in blood pressure. D. 3 cc/kg additional in divided doses—progressive fall in blood pressure and followed by death.

6 mg of either isomer in a 30 cc bath caused a prompt depression of the frequency and amplitude of contraction. This action was not shared by the thiocyanate ion. Fig. 2 shows the action of ortho-thiocyanobenzoic acid on the rat's uterus; 12 mg of the ortho isomer elicited an activity comparable to 6 mg of the para compound.

FIG. 2.

Effect of o-thiocyanobenzoic acid on the rat's uterus. At each arrow 3 mg was added to bath.

Acute toxicity—white rat. Acutely fatal intraperitoneal doses of the ortho-, meta- and para-thiocyanobenzoic acids elicited hyperirritability, hyperreflexia, tremors and transient convulsive seizures. This syndrome was followed rapidly by weakness of the extremities and flaccidity. The animals died of respiratory failure. The heart continued to beat for some time after respiratory collapse. Fatal doses usually killed within 30 minutes. The LD_{50} upon intraperitoneal injection into the white male rat (3 hours) for each of the 3 isomers is ortho 32 mg/kg, S.E. $= \pm 2.1$ mg; meta 17 mg/kg, S.E. $= \pm 1.3$ mg; para 22 mg/kg, S.E. $= \pm 1.9$ mg. For sodium thiocyanate the LD_{50}, under these conditions, is 540 mg/kg, S.E. $= \pm 42.5$ mg.

Effect on brain dehydrogenases. A suspension of rat's brain brei supplied the tissue dehydrogenase systems for these studies. The Thunberg methylene blue reduction technic was employed. The meta- and para-thiocyanobenzoic acids were shown to inhibit the dehydrogenase systems. Ortho-thiocyanoben-

TABLE I.
Effect of Isomeric Thiocyanobenzoic Acid and the Thiocyanate Ion on Cytochrome Oxidase.

Compound	Effective conc. for cytochrome oxidase inactivation, mg %
Sodium thiocyanate	5000
Ortho-thiocyanobenzoic acid	300
Meta- " "	6
Para- " "	0.18

zoic acid shared this activity. In concentrations of from 1-7000 to 1-1000 the decolorization time for methylene blue was approximately doubled. This property is not shared by the thiocyanate ion(1).

Effect on cytochrome oxidase. A suspension of rabbit's brain brei supplied the cytochrome oxidase. Its activity was measured by the Nadi reaction. The reaction was allowed to proceed for 5 minutes, at 25°C. At the end of this period the absence of color compared with a control was considered inactivation of the oxidase.

These studies show that the thiocyanate ion is ineffective in inactivating cytochrome oxidase. Ortho-thiocyanobenzoic acid exhibits little inhibition of cytochrome oxidase activity. The para compound, however, shows a powerful nullifying action on cytochrome oxidase. This compound was shown to be free from any contamination with cyanide by chemical means.

Discussion. The thiocyanate radical attached to a phenyl group is a potent depressor agent, if the radical is meta or para, to the carboxyl group. The ortho compound does not share this activity. The relaxant action of the three isomeric acids on smooth muscle appears to be of the same order of magnitude. Brain reductase systems are inactivated by small concentrations of each of the isomeric acids. However, cytochrome oxidase is inactivated by the meta and para compounds but is not strongly affected by the ortho acid. Indeed on the blood pressure and on cytochrome oxidase the action of the ortho acid is somewhat similar to the thiocyanate ion and markedly dissimilar to the meta- and para-thiocyanobenzoic acids.

Summary. 1. The pharmacologic responses to the three isomeric thiocyanobenzoic acids have been investigated. 2. The meta and para compounds are powerful depressors. This action is not shared by the ortho compound. 3. p-Thiocyanobenzoic acid inactivates cytochrome oxidase in concentrations similar to cyanide. The meta compound is less active and the ortho isomer exhibits little inhibition. 4. All three isomers are relaxants to smooth muscle.

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Increases in Serum Thyroxin during Uncomplicated Pregnancy.* (17892)

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It has been established that an increase in serum precipitable or protein-bound iodine to levels above the euthyroid range of 4 to 8 γ % frequently occurs during pregnancy in human subjects(1). The studies herein reported indicate that the serum thyroxin also rises.

Materials and methods. Sera have been obtained at random for analyses from 57 gravid women usually during the second or third trimester. With the exception of 4 under the care of private physicians all were regular visitants to the dispensary of the Elizabeth Steel Magee Hospital. In each

* Aided by a grant from the Committee on Growth, National Research Council.

1. Heinemann, M., Johnson, C. E., and Man, E. B., *J. Clin. Invest.*, 1948, v27, 91.

TABLE I.
Circulating Protein-bound Iodine (PBI) and Thyroxin Are Increased in Pregnancy.

Group	No. of subjects	No. of sera*	PBI (Mean ± S.D.) γ %	No. of subjects	No. of sera*	Thyroxin (Mean ± S.D.) γ %
Control†	25	89	5.4 ± 0.7	23	23	3.7 ± 0.8
Pregnant	57	81	7.8 ± 1.3‡	21	23	5.4 ± 1.0§

* Analyzed in duplicate or triplicate.

† Consisting of healthy adults, males and non-pregnant females, since no statistically significant difference could be demonstrated in their protein-bound iodine and thyroxin concentrations.

‡ Significantly different from control group since "t" was found to be 11.57 and "p" = <0.0001.

§ As in preceding footnote with "t" = 4.76 and "p" <0.0001.

instance pregnancy proved to be free from major complications and terminated in the delivery of a living child at term. A total of 81 sera have been analyzed in duplicate or triplicate for the concentration of protein-bound or serum precipitable iodine by the method of Barker as used in this laboratory(2,3). In 43 of the patients blood was withdrawn for analysis only once. In the remaining 14 repeat samples were obtained one or more times during gestation. In view of the variable relationships between the serum proteins and protein-bound iodine(4), albumin and globulin concentrations(5,6) have been measured in all but a few of the pregnant subjects. In 23 sera from pregnant women with protein-bound iodine concentrations within and above the euthyroid range thyroxin levels were also measured using the method of Taurog and Chaikoff(7). For purposes of comparison thyroxin and protein-bound iodine analyses have been conducted in healthy non-pregnant adult females and males. The results are presented in Table I.

Results. A. *Protein-bound iodine levels.* The average value for this fraction in pregnancy proved to be significantly higher, statistically, than the mean recorded in non-pregnant adults (Table I). These results agree closely with the findings of others and support the conclusion that uncomplicated pregnancy is usually associated with a rise in the serum precipitable or protein-bound iodine(1). In 28 of the patients in our series the increase was of sufficient magnitude to place the protein-bound iodine above the upper limit of the range characteristic of euthyroid non-pregnant adults(8), 8.0 γ %. The highest value observed was 11.3 γ %. There were no discernible clinical signs of thyrotoxicosis in this subject nor in those with lesser elevations in protein-bound iodine. The results of serial analyses indicate that this rise is not necessarily stable. At times slight but probably definite increases or decreases were subsequently observed, while in others previous values were reduplicated. This contrasts with the relative constancy of this serum iodine fraction in non-pregnant subjects(9). In the patients in whom longer periods of observation are available there was evident a distinct trend upward during successive analyses. The recorded rises were not always of sufficient magnitude however to place the protein-bound iodine value above euthyroid limits.

B. *Thyroxin concentrations.* From Table I it is apparent that the serum thyroxin level measured usually in triplicate during pregnancy ranged between 3.4 and 6.9 γ %,

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5. Majoor, C. L. H., *J. Biol. Chem.*, 1947, v169, 583.

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9. Danowski, T. S., Hedenburg, S., and Greenman, J. H., *J. Clin. Endocrinol.*, 1949, v9, 768.

averaging 5.4 ± 1.0. This differs significantly from the mean of 3.7 ± 0.8 γ % recorded in non-pregnant control subjects. These values represent the actual results of analyses. They have not been corrected for the loss, approximating 10%, observed in thyroxin recovery studies in this and in other laboratories(7), because of the uncertainty of its magnitude in serum analyses. Such a correction however would only serve to emphasize the validity of our findings.

A good correlation is evident between the level of thyroxin and of protein-bound iodine in pregnancy, represented in the line of best fit $y = 1.0008 + 0.5741x$, where y indicates thyroxin and x the protein-bound iodine. The standard error of estimate of "y" proved to be 0.667, and the correlation coefficient, +0.734.

Discussion. The reality of the rise in circulating protein-bound iodine during uncomplicated pregnancy in human subjects has been confirmed in these studies. It has also been shown that this rise is associated with a significant increase in the serum thyroxin which is of course a subdivision of the protein-bound iodine. It seems reasonable to suggest furthermore that changes more marked than these may occur in pregnancy since in most of our studies serum specimens were obtained at random and hence probably do not represent the maximal values attained. These findings invite speculation concerning factors possibly operative in inducing these changes. Excluding artefacts such as those induced by administering organic iodine dyes (10) the concentrations of circulating thyroxin and protein-bound iodine can be affected by (a) the rates at which these compounds are synthesized, (b) the rates at which they are released from their sites of origin, (c) the characteristics of the plasma transport system, and (d) the rates at which they are metabolized or excreted. No data are available which conclusively implicate or exclude any of these factors in the changes which have been observed. Although it has been shown in animals that the thyroidal uptake of radioactive iodine is increased during pregnancy, no comparable studies in humans have been reported(11). Furthermore, though it is true that an increased intake of exogenous inorganic iodide raises the protein-bound and thyroxin iodine in the gland(12) as well as the circulating inorganic and protein-bound iodine(13), there is no evidence that pregnancy is necessarily associated with a higher ingestion of iodine or iodide. The persistence of the elevations in thyroxin and protein-bound iodine makes it quite improbable that the observed changes represent merely an increase in the rate of discharge of stored thyroid gland products. On the other hand it is possible that during gestation the plasma proteins bind thyroxin and organic iodine in larger quantities than usual. In our series of patients, the concentrations of serum albumin and globulin were 3.55 ± 0.42 and 3.20 ± 0.34 g %. In keeping with the findings of others these averages are lower and higher, respectively, than those characteristic of non-pregnant control subjects(14). If it is accepted that the organic iodine of serum is bound chiefly to albumin, one would then expect a fall rather than a rise in protein-bound and thyroxin iodine. It is by no means certain however that serum albumin and protein-bound iodine levels are so directly related(4,15). It is possible therefore that protein fractions do bind more organic iodine during pregnancy. There is no evidence for or against the possibility that in these patients the excretion of iodine compounds through the liver, gastro-intestinal tract or other routes(16,17) is diminished. Moreover, the known elevation in basal metabolic rate which usually occurs in the last

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16. Gross, J., and Leblond, C. P., *Fed. Proc.*, 1949, v8, 62.

trimester(18), does not resolve any of the uncertainties which have been cited, since (a) the rise in protein-bound iodine can be demonstrated in the first trimester(1) and (b) euthyroid protein-bound iodine values have been recorded in our series during the last trimester. It is obvious that these questions can probably be answered by studies which define the rates of production, release, and degradation of thyroxin and related compounds. In conducting such studies with radioactive tracers it must be kept in mind that the fetal thyroid in humans assimilates iodide from the 12 to 16 week point onward (19). Finally, though it is not apparent which of the above factors are present in the observed findings, it seems reasonable to suspect that they are mediated through hormonal changes occurring during pregnancy.

Summary and conclusions. The rises in protein-bound iodine which usually occur in uncomplicated pregnancy in human subjects are associated with an increase in the thyroxin fraction of the protein-bound iodine.

The authors are indebted to Mr. Carroll S. Weil for assistance in the statistical evaluation of these data.

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Differentiation between Bacterial and Tryptic Gelatin Liquefaction as Aid to Diagnosis of Fibrosis of Pancreas. (17893)

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The failure of fecal specimens from patients with cystic fibrosis of the pancreas to cause liquefaction of the gelatin coating on x-ray film, in contrast to specimens from normal children or patients suffering from other maladies, has been shown by Schwachman(1,2) to be a useful aid to the diagnosis of this pancreatic disease. The test is based on the fact that in cystic fibrosis of the pancreas trypsin is absent or present in subnormal amounts in the feces. As shown by Johnstone(3), cultures of certain bacterial species, particularly members of the genera *Proteus* and *Pseudomonas*, may also cause a positive x-ray film test. These microorganisms are not rarely encountered in the feces of infants and young children(4) and their presence may be the cause of false positive x-ray film liquefaction tests. An attempt was made, therefore, to develop a test which may differentiate between gelatin liquefaction due to pancreatic trypsin and that due to bacterial enzyme.

Material and methods. The x-ray film liquefaction test as used for the diagnosis of cystic fibrosis of the pancreas has been previously described(2,3). Since Eastman Kodak "Blue Brand" x-ray film proved to be more sensitive than either Dupont "Xtra Fast Medical" x-ray film or Hammer panchromatic portrait photographic film for this test, the former was used throughout this investigation. Soybean trypsin inhibitor was procured from Worthington Biochemical Laboratory (Freehold, N. J.) and was dis-

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3. Johnstone, D. E., *Pediatrics*, in press.

4. Neter, E., and Clarke, P., *J. Ped.*, 1945, v26, 390.

TABLE I.
Comparative Effects of Enzyme Inhibitors on X-ray Film Liquefaction by Feces and Broth Cultures of Gelatin-liquefying Bacteria.*

	Broth cultures			Feces		
		No. of strains tested			No. of specimens tested	
Inhibitor	Dilution	Inhibited	Not inhib.	Dilution	Inhibited	Not inhib.
Soybean trypsin inhibitor (2 mg/ml)	1:5	0	69	1:25	41	3
	1:50	0	69	1:50	107	0
Zinc oxide (2% suspension)	1:5	25	15	1:25	0	43
	1:50	39	1	1:50	0	43
	1:100	40	0			
Zinc peroxide (3% suspen.)	1:5	32	0	1:5	1	32
	1:50	32	0	1:25	21	7
	1:100	32	0	1:50	21	7
Sodium perborate (4% sol.)	1:5	18	19	1:5	0	43
	1:50	32	5	1:25	0	43
	1:100	37	0	1:50	0	43

* 38 strains of *Pseudomonas aeruginosa*, 26 strains of *Proteus ammoniae*, 2 strains of *Proteus morganii*, and 3 strains of other pseudomonas.

solved in physiological saline. Various freshly isolated strains of *Proteus ammoniae, Proteus morganii,* and *Pseudomonas aeruginosa* were grown in brain heart infusion for 24 hours. Fecal specimens were obtained from healthy children and adults as well as from children suffering from diseases other than cystic fibrosis of the pancreas.

Results. On the assumption that gelatin liquefaction by the above mentioned bacteria is due to an enzyme or enzyme system other than trypsin, an attempt was made to differentiate between this bacterial enzyme and pancreatic trypsin by the use of a trypsin inhibitor. The action of soybean trypsin inhibitor on 69 strains of x-ray film-liquefying bacteria was tested. Serial dilutions of 24-hour broth cultures of these microörganisms (vol. 0.2 ml) were mixed with equal amounts of (a) soybean trypsin inhibitor containing 2 mg per ml in one series and (b) physiological saline solution in the other series serving as controls. A drop of each mixture was then placed on x-ray film, incubated, and, one hour later, the presence or absence of gelatin liquefaction was noted. The results are summarized in Table I and indicate that soybean trypsin inhibitor regularly failed to prevent x-ray film liquefaction by the strains tested. In contrast, as may be seen from this table, when 107 fecal specimens obtained from both children and adults were properly diluted (1:50) in physiological saline and mixed with soybean trypsin inhibitor in concentrations of 2 mg per ml, inhibition of gelatin liquefaction occurred without exception, indicating that the gelatin liquefaction in this latter group was due to the presence of trypsin.

A search for a compound which inhibits the gelatin liquefying enzyme of bacteria to a greater extent than pancreatic trypsin was made among compounds which are oxidizing agents, contain heavy metal ions, or which are known to inhibit certain enzymes. The following compounds were tested: potassium permanganate, BAL (2, 3 dithiopropanol), Lugol's solution, potassium ferricyanide, sodium perborate, zinc oxide, zinc peroxide, ammonium perchlorate, boric acid, potassium persulfate, oxalic acid, sodium arsinate, strontium bromide, bismuth subiodide, mercury oxycyanide, mercury potassium iodide, hydrogen peroxide, methylene blue, iodoacetic acid, merthiolate, mercurochrome, zephiran, mercresin, sodium fluoride, metaphen, argyrol and neosylvol.

As may be seen from Table I, a 2% suspension of zinc oxide, 3% suspension of zinc peroxide, and 4% solution of sodium perborate, all in physiological saline, inhibit gelatin liquefaction by the above strains of microörganisms far more effectively than gelatin liquefaction resulting from the action of fecal specimens in suitable dilutions. Similarly, these chemical compounds failed to inhibit gelatin liquefaction by a 1:50 saline solution of crystalline trypsin, whereas the soybean trypsin inhibitor completely inhibited this solution. The other compounds tested were less satisfactory for the differentiation between bacterial and pancreatic gelatin liquefying enzymes.

Discussion and summary. In order to render the x-ray film liquefaction test by fecal specimens more informative it is highly desirable to be able to differentiate between gelatin liquefaction due to the activity of pancreatic trypsin and that due to the action of bacterial enzyme. Soybean trypsin inhibitor, which prevents the liquefaction of gelatin (x-ray film) by crystalline trypsin and trypsin in feces, fails to inhibit gelatin liquefaction by various bacteria, including *Proteus ammoniae, Proteus morganii,* and *Pseudomonas aeruginosa.* Suitable preparations of zinc oxide, zinc peroxide, and sodium perborate have no or only a slight effect on trypsin in feces but markedly inhibit the bacterial enzyme responsible for gelatin liquefaction.

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Relative Efficacy upon *Pasteurella multocida* of Various Antibiotics Aureomycin, Terramycin, Bacitracin, and Polymyxin B. (17894)

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Recently a strain of *Pasteurella multocida* was isolated from an abscess at the nose of a boy 15 years of age. The infection developed subsequent to a plastic operation undertaken to correct a deformity resulting from "a kick of a horse to the nose". Since this pathogen could be demonstrated repeatedly over a period of 6 months and other disease-producing microörganisms were not recovered, the conclusions are drawn that the infection was caused by the pasteurella organism, was of animal origin, and had remained latent until activated by the operation. That human infections with this microörganism have been reported only infrequently is evident from a review of the world literature by Schipper(1), who collected but 39 authentic cases described between 1930 and 1947. Because of the lack of data on the effectiveness of some of the newer antibiotics upon this microbe, a study was undertaken to determine the relative efficacy of 7 antibiotics upon 8 strains of *P. multocida,* including the one isolated from the above patient. The results are embodied in this report.

Materials and methods. The following strains of *P. multocida* were employed:

No.	Strain	Source
200	*P. avicida*	*
1	Bovine	†
2	Unknown	†
3	Canine	†
9656	*P. oviseptica*	‡
9658	" "	‡
10544	From sputum	‡
4392	" abscess	§

* Cornell University (Dr. Brunner).
† Iowa State College (Dr. Merchant).
‡ American Type Culture Collection.
§ Children's Hospital, Buffalo.

The strains were maintained on blood agar and were transplanted twice a week. Six antibiotics were employed. Penicillin-G, crys-

1. Schipper, G. J., *Johns Hopkins Hosp. Bull.,* 1947, v81, 333.

TABLE I.
Minimal Growth Inhibitory Concentrations of Sulfadiazine and Various Antibiotics on *Pasteurella multocida.*

Drug	Strain							
	1	2	3	200	9656	9659	4392	10544
Sulfadiazine, mg %	.003-10	.01-10	.01-10	.01-10	.03-10	.03-10	.01-10	.03-10
Penicillin, units %	.03-1	.1-1	.01-1	.03-3	.1-1	.1-1	.3-1	.03-10
Streptomycin, μg %	1-10	1-10	10-10	10-10	1-10	1-10	10-10	10-10
Aureomycin, μg %	.01-1	.1-1	.1-3	.01-1	.25-1	.1-1	.01-0.1	.3-1
Chloromycetin, μg %	.1-1	.3-1	.3-1	.01-1	.3-1	.3-1	1-10	.3-1
Bacitracin, units %	10-100	3-100	10-100	10-100	10-30	3-100	.3-30	10-100
Polymyxin, μg %	.01-5	.1-3	.03-3	.03-1	.003-3	.0001-3	.1-10	.1-10
Terramycin, μg %	.3-1	.3-1	.3-1	.3-1	.3-1	1-3	.1-1	.3-1

talline-potassium, dihydro-streptomycin hydrochloride, and bacitracin were procured from commercial sources. Aureomycin hydrochloride (intravenous) was made available through the courtesy of Dr. B. W. Carey of Lederle Laboratories; pure crystalline chloramphenicol (chloromycetin) was kindly supplied by Dr. J. P. Gray of Parke, Davis and Co.; polymyxin B sulfate ("Aerosporin") by Dr. D. S. Searle of Burroughs Wellcome & Co. and terramycin hydrochloride by Dr. Elliott R. Weyer of Chas. Pfizer & Co. The drugs were dissolved in various concentrations in brain heart infusion (volume 1.8 ml) and, together with broth used as control, seeded with 24 hours broth cultures of the above strains. The size of the inocula was 0.2 ml and contained either 1 to 100 million organisms per ml or 100 to 1,000 organisms per ml. The tubes were incubated at 37°C and the resulting growth was noted at regular intervals.

Results. The minimal amounts of the 7 antibiotics capable of suppressing visible growth for 24 hours of each of the 8 strains of *P. multocida* was determined and the results were summarized in Table I. At least 6 experiments were carried out for each drug concentration and for each strain. The first figure given for each drug concentration is the one obtained in tests using small inocula and the second figure the one observed with large inocula of the particular strain.

Discussion. Studies on the antibacterial activity of several antibiotics against *Pasteurella multocida* have been reported previously, but different technics and different strains were used in each investigation. It was deemed of interest, therefore, to determine the relative efficacy of these antibiotics, using a single method and identical strains, and to explore the efficacy of 3 antibiotics, aureomycin, bacitracin, and terramycin whose antibacterial activity against this species has not been studied before. It is evident from Table I that aureomycin is highly effective against all 8 strains of *P. multocida.* The strain (No. 4392) isolated from the above mentioned patient was sent to Lederle Laboratories and proved to be the most susceptible gram-negative organism Dr. Dornbush (2) has encountered up to that time. Terramycin, which was described recently by Finlay and associates(3) as an antibiotic of potential clinical usefulness, also inhibits the growth of this species. *P. multocida* appears to be as highly or more susceptible to this antibiotic as the various bacterial species tested by Finlay *et al.* although it must be realized that different methods were employed in these investigations. Bacitracin was found to be relatively ineffectual against *P. multocida,* particularly when one calculates the amounts of drug necessary to inhibit growth. Based on the fact that 100 units of the preparation used represent approximately 2,000 μg it is evident that, with the exception of one strain, from 70 to 2,000 μg/ml was required to effect bacteriostasis. This finding is not surprising in view of the fact that

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bacitracin is largely effective against gram-positive microörganisms, but it is interesting to point out that penicillin, whose spectrum of antibacterial activity somewhat resembles that of bacitracin, was found to be highly effective against *P. multocida,* as may be seen from Table I. That penicillin inhibits growth of this species was previously reported by Schipper(1) and by Queen and Quortrop(4). In view of the fact that *P. multocida* is a gram-negative rod of the family *Parvobacteriaceae* which includes, among others, the members of the genus *Hemophilus* it is somewhat surprising to find that 8 strains of *P. multocida* are rather resistant to streptomycin, as may be seen from Table I. Coles(5) studied the sensitivity of 78 strains of *P. multocida* by the agar plate method and found that for complete inhibition all but seven strains required 1 μg or more and that none of the strains was inhibited by less than 0.5 μg of streptomycin.

The above reported experiments confirm the observation of McLean, Schwab, Hillegas and Schlingman(6) and of White, Alverson, Baker and Jackson(7), to the effect that both chloromycetin and polymyxin are effective against *P. multocida.* The minimal effective concentration of polymyxin and "Aerosporin", according to White and associates(7), was 1 μg/ml. It may be pointed out that as little as 0.0001 to 0.03 μg/ml was adequate to prevent growth of small inocula of five of the strains of *P. multocida* used in this study.

Summary. The bacteriostatic activity toward eight strains of *Pasteurella multocida* of aureomycin, terramycin, and bacitracin and the relative efficacy of four additional antibiotics, previously studied only singly, have been determined. It was found that the eight strains were very susceptible to penicillin, aureomycin, chloromycetin, terramycin and polymyxin B but rather resistant to bacitracin and streptomycin.

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Eosinophil Response to Epinephrine and Nor-epinephrine. (17895)

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It has been demonstrated repeatedly that a variety of stress conditions(1), adrenocorticotrophic hormone, adrenal cortical hormone and epinephrine(2,3), produce a pronounced reduction in the number of circulating eosinophils. This response has been shown to depend on the stimulation of the adrenal cortex. The fall in circulating eosinophils is so constant and sensitive an indicator of oxycorticosteroid release that it has been incorporated into a standard test for adrenal cortical function(4). For this purpose, either ACTH or epinephrine has been used. Nor-epinephrine†

* Sponsored by Veterans Administration and published with approval of Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect opinion or policy of Veterans Administration.

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is 1(3',4'-dihydroxyphenyl)-2-aminoethanol and differs from epinephrine only in the absence of a methyl group on the terminal nitrogen. It has been found in the normal adrenal medulla, in the post-ganglionic adrenergic nerve endings and in high concentration in pheochromocytomas(5). Nor-epinephrine has been postulated by some to be the mediator of the excitor impulse of the sympathetic nervous system(6,7). Despite the remarkable similarity in chemical configuration of the two sympathomimetic amines there is a pronounced difference in their pharmacologic activity(8). The purpose of this study was to compare the effect of nor-epinephrine and epinephrine on the circulating eosinophils of man.

Material. A group of hospital patients admitted for a variety of complaints, but generally in good health, was selected for this study. No patient selected was suffering from obvious adrenal insufficiency, heart disease or any chronic debilitating disease.

A total of 53 patients was grouped for study as follows: 22 patients received 0.2 mg of epinephrine. 20 received 0.2 mg of nor-epinephrine. 11 were given 0.3 mg epinephrine; 6 received 0.3 mg of nor-epinephrine at later date

Method. Solutions of epinephrine and nor-epinephrine were prepared to contain 0.2 mg or 0.3 mg of the material in 200 ml of normal saline. Only the levo form of nor-epinephrine was used in this study. The test solutions were administered intravenously during the course of 1 hour. The tests were done at 8:00 A.M. on subjects who were kept at basal conditions from 10:00 P.M. of the preceding night. Venous blood was drawn immediately before starting the infusion and

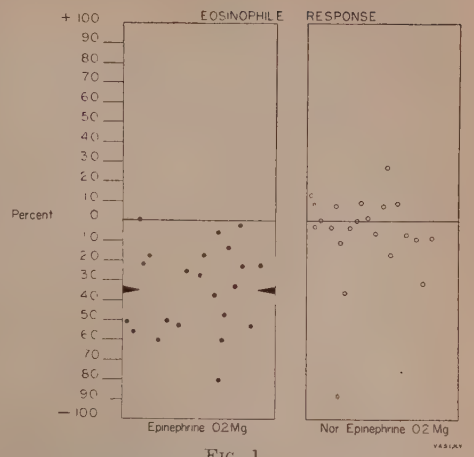

FIG. 1.

% change in eosinophils following 0.2 mg of epinephrine and nor-epinephrine.

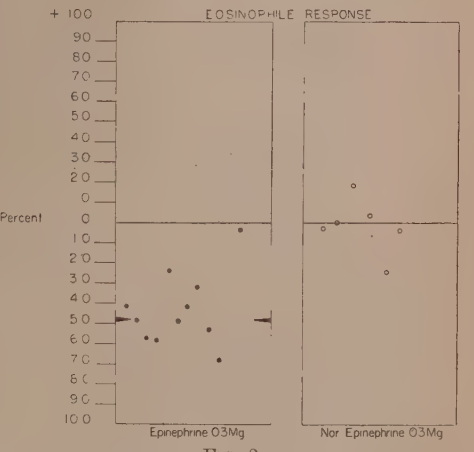

FIG. 2.

% change in eosinophils following 0.3 mg of epinephrine and nor-epinephrine.

4 hours later. Five ml samples of venous blood were taken on each occasion, without stasis, and stored in oxalate (Wintrobe) tubes at 4°C. The enumeration of the eosinophils was performed by the hemocytometer technic, using the 0.2 mm deep Levy counting chamber. The diluting fluid used was that described by Randolph(9). Two complete chambers were counted for each determination.

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Results. There was an average fall of 35% in the circulating eosinophils 4 hours after the administration of 0.2 mg epinephrine. In 50% of the observations the fall in eosinophils was greater than 30%. On the whole, a consistent fall in the number of eosinophils was not noted following the administration of 0.2 mg of nor-epinephrine with the exception of two subjects who showed drops of 36% and 31%. (Fig. 1). An average fall of 48.6% in the circulating eosinophils was found after the administration of 0.3 mg of epinephrine. One subject with schistosomiasis and a high initial eosinophil count (418 cells per cu mm) showed no significant change. Following the injection of 0.3 mg nor-epinephrine no significant change in the eosinophile count was found. One patient responded with a drop of 23%. (Fig. 2).

Many of the subjects receiving 0.3 mg of epinephrine complained of mild palpitation, tremor and tingling. The majority of the patients receiving nor-epinephrine complained of mild pressure headaches and demonstrated a moderate bradycardia.

It was our impression that the 3 patients who, following the administration of nor-epinephrine, responded with a drop in the circulating eosinophils, were all tense, anxious, and emotionally disturbed by the experimental procedure. No other obvious factors were in operation.

Conclusion. (1) Nor-epinephrine (levo) in dosage given (0.2-0.3 mg) produces no consistent change in the number of circulating eosinophils of man.

(2) Nor-epinephrine is probably not a physiologic excitor of the pituitary adrenal system.

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Sex Difference in the Response of Rats to Sodium Pentobarbital.* (17896)

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Considerable difference of opinion has been expressed in the literature concerning the part played by sex in the response of the rat to anesthetic doses of sodium pentobarbital (Nembutal). Thus Barron(1) and Carmichael(2) found this anesthetic equally effective in both sexes, while Holck and Kanan(3) found that males were more resistant to its action. This resistance was confirmed by Moir(4) and Homburger *et al.*(5) in adult males, but the difference between the sexes disappeared or was actually reversed in immature animals. These reports are based upon "sleeping time" following single doses of sodium pentobarbital given once or repeated upon successive days. In the course of a series of experiments on neuromuscular function, the opportunity arose of examining this problem from a different point of view. The necessity for maintaining deep surgical anesthesia over periods of several hours served to sharpen the contrasting response of the two sexes to the narcotic. The size of the series was sufficient to allow statistical analysis.

The rats used were adults of the Sherman and Whelan strains weighing from 160 to 420 g. They were fed on a diet of Purina

* Supported in part by a contract between the Office of Naval Research, Department of the Navy, and the Johns Hopkins University.

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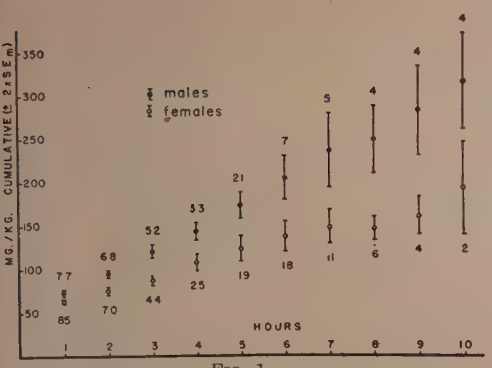

FIG. 1.

pellets with unlimited water intake. Each received an initial intraperitoneal injection of sodium pentobarbital, 50 mg/kg freshly prepared in 2.5% solution in distilled water. Tracheotomy was performed and, in most instances, one hind leg was prepared for the recording of muscle twitches or action potentials. Since skin incisions and nerve sections were required, a deep stage of surgical anesthesia was maintained. Depth of narcosis was gauged at frequent intervals by pinching the animal's tail with forceps. If there occurred a sharp expiratory movement, consisting of explosive contraction of both abdominal and chest walls, a supplementary intraperitoneal injection of 2.5 to 7.5 mg was given. With this schedule it was possible to maintain relatively even anesthesia over prolonged periods.

It soon became evident that males required noticeably larger total doses, *i.e.* more frequent injections, if the desired depth was to be smoothly maintained. The results are presented graphically in the figure. Each point represents the average of cumulative doses at the end of the hour indicated by the abscissa, zero being the time of the original injection of anesthetic. The vertical bars represent twice the standard error of the mean. The figures above and below these symbols are the numbers of animals of each sex in each hourly group. The series decreases in size with time because of the termination of the neuromuscular experiments from which the anesthesia figures were derived. Despite the small numbers available in the later hours, the difference between the two sexes continues to show high statistical significance. An attempt was also made to rule out unconscious weighting of the curves. Three male and 3 female animals were dressed in cloth bags in a manner effectively concealing their sex from the experimenter. Observations were then made simultaneously on the group. The difference between the sexes noted in the larger series was again apparent. It is also noteworthy that over half of the total series was accumulated before attention was directed to the observation here reported. Since the average adult male rat is considerably heavier than the female of comparable age, the results were also analyzed according to weight. Spot diagrams showed no correlation between weight and utilization of the drug.

No attempt has been made to explain this difference in anesthetic response of the 2 sexes. The studies of others have demonstrated that castrated males tend toward the female sensitivity to sodium pentobarbital, and that this effect may be at least partially reversed with androgens(6,7). It has also been noted that sex difference is demonstrable only with certain barbiturates, and that these are all short-acting drugs detoxified in the liver(7). Since the curves presented herewith are very nearly straight lines with a common origin at 50 mg/kg, it appears likely that the initial anesthetic process is similar for all adult rats, but that detoxification of the narcotic proceeds at a constant but different rate in each sex.

Conclusions. Adult male rats require significantly larger amounts of sodium pentobarbital than do females for the maintenance of deep surgical anesthesia.

We wish to thank Dr. Rowland V. Rider, statistician, The Johns Hopkins Hospital, for assistance in the statistical treatment of the data.

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Biocytin in Bacterial Deamination of Aspartic Acid. (17897)

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Lichstein and co-workers(1-4) reported that the aspartic acid, serine, and threonine deaminase activity of certain bacteria may be inactivated by exposure of the cells for a short time to pH 4 in M phosphate buffer. The deaminase activity of such inactivated cells could be restored with biotin, adenylic acid, or yeast extract. Yeast extract was found to be particularly active in stimulating the system. Lichstein reported (3) that yeast extract is approximately 100 times more active than can be accounted for on the basis of its biotin content. It was concluded that there exists in yeast extract a more highly active, presumably coenzyme, form of biotin that yields biotin on hydrolysis. A preliminary concentration of the so-called coenzyme of aspartic acid deaminase has been described(5). Wright *et al.*(6) have confirmed Lichstein to the extent that they showed that bacterial preparations with a reduced ability to deaminate aspartic acid can be obtained and that, when the deaminase reaction is run at pH 7, after inactivation of the cells at pH 4 in M phosphate buffer, activity can be restored by either biotin or yeast extract. Attempts at reactivating cells with biotin when the deaminase reaction is run at pH 4 in M/2 phosphate buffer, such as described by Lichstein in his paper(3) demonstrating special activity for yeast extract, have been unsuccessful. In contrast to Lichstein and coworkers, when the amounts of biotin and yeast extract used to stimulate deamination at pH 7 were titrated downward, biotin usually was found to be more active in the system than an equivalent of biotin as it occurs in yeast extract.

Recently Wright *et al.*, have announced(7) the isolation in crystalline form of a component of yeast extract, termed biocytin, that is available as a source of biotin to *Lactobacillus casei* but not to *Lactobacillus arabinosus*. Acid or alkaline hydrolysis yields as one moiety biotin or its microbiological equivalent for all biotin-requiring microorganisms studied. Chromatographic studies have failed to demonstrate the existence in yeast extract of more than two forms of biotin, namely free biotin and biocytin.

A biocytin concentrate has been furnished to Dr. Lichstein who has reported(8) that biocytin is inactive in stimulating the aspartic acid deaminase activity of cells that have been exposed to pH 4 M phosphate buffer. Studies reported in this paper with crystalline biocytin demonstrate that under the experimental conditions recently described(6) biocytin may be shown to have activity. The activity of biocytin has varied with various bacterial preparations used from essentially no activity to activity equal to that shown by biotin itself.

Experimental. Procedures that have been employed in the aspartic acid deaminase experiments have been described in detail(6). The biocytin used was crystalline material (7) and contained no free biotin by microbiological assay with *Lactobacillus arabinosus* (9).

Results and discussion. The results that have been obtained are summarized in Table

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3. Lichstein, H. C., *J. Biol. Chem.*, 1949, v177, 125.
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TABLE I.
Summary of Aspartic Acid Deaminase Experiments.

Exp. No.	Organism	pH of deaminase reaction	Ammonia N found per tube		
			No supplement, γ	Biotin, γ	Biocytin, γ
1	*Proteus vulgaris*	7	9.	13.6 (.001 γ)	—
2	,, ,,	7	5.4	11.1 (.001 γ)	—
3	,, ,,	7	5.4	9.0 (.001 γ)	7.00 (.001 γ)
4	,, ,,	7	3.7	6.6 (.001 γ)	4.5 (.001 γ)
5	,, ,,	7	7.5	34.5 (.001 γ)	36.7 (.001 γ)
6	,, ,,	7	5.8	31.0 (.0001 γ)	28.5 (.0001 γ)
				28.5 (.001 γ)	26.3 (.001 γ)
7	,, ,,	7	11.	20.5 (.00001 γ)	18.7 (.00001 γ)
				33.0 (.0001 γ)	31.5 (.0001 γ)
8	,, ,,	7	17.2	29.0 (.001 γ)	25.8 (.001 γ)
9	,, ,,	4	0.	0. (.001 γ)	0. (.001 γ)
10	*Escherichia coli*	7	10.8	11.8 (.001 γ)	15.2 (.001 γ)

Figures in parentheses indicate micrograms of biotin or biocytin calculated as biotin per tube.

I. As previously reported, it has been impossible to demonstrate stimulation of aspartic acid deaminase with biotin at pH 4. At pH 7 activity is demonstrable with both biotin and biocytin. There is a suggestion that biocytin is less active than biotin in those instances where relatively small amounts of ammonia are produced with biotin (see Exp. 3 and 4). In those instances where relatively large amounts of ammonia are produced with biotin, biocytin has activity equal to that of biotin (see Exp. 6 and 7). Thus it would appear that the ability to utilize biocytin may be a function of the metabolic activity of the bacterial preparation employed.

In no experiment has biocytin shown significantly greater activity in stimulating the aspartic acid deaminase system than that obtained with biotin. Since chromatographic data have failed to show the existence of more than 2 forms of biotin in yeast extract, these data would indicate that, if a highly active coenzyme for the aspartic acid deaminase system exists, biotin and/or biocytin is concerned with its formation rather than as one of its components.

Summary. Biocytin, like biotin, has been shown to be active in stimulating the aspartic acid deaminase system of bacterial cells that have been inactivated by exposure to pH 4 M phosphate buffer.

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Presence of Secretin in Cystic Fibrosis of the Pancreas. (17898)

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The existence of unusually viscid duodenal secretions in cystic fibrosis of the pancreas has long been recognized. The explanation has been uncertain but among others, a hyperparasympathetic state and a defect of mucin liquefaction have been prominently considered. Baggenstoss, Power and Grindlay have recently reported the absence of secretin in the small bowel of a fibrocystic case(1) and hypothesized that a congenital absence or deficiency of this hormone might be the etiological factor(2). Interest in this possibility has been keen since it suggested a therapeutic approach to this baffling disease. In the present study, the distribution of secretin was determined in a variety of control infants and children as well as in 4 with fibrocystic disease of the pancreas. In 2 of the latter (SB and PO; Table II), the clinical diagnosis had been verified antemortem by duodenal drainage, whereas this was not performed in the others, due to the debilitated states of the infants. However, when first seen by the present authors, the cough, pneumonitis and steatorrhea were typical. Stool trypsin was absent in GC and was not tested in CW because of pancreatin administration. The autopsy diagnosis was definite in all 4.

The first 3 feet of jejunum (together with some duodenum in a few cases) were removed at autopsy, everted and extracted with 100-120 ml of 0.4% hydrochloric acid. Saturation of the acid extract with sodium chloride led to the separation of the 'A-precipitate' which was then submitted to trichloroacetic acid treatment according to the procedure of Greengard and Ivy(3). The resulting SI concentrates were assayed for secretin in anesthetized dogs by the method of Gershbein, Wang and Ivy(4). The log dose-response curves for a number of the dogs, served as invaluable tools for potency evaluation. Baggenstoss, Power and Grindlay also prepared SI concentrates from the small bowel of controls and the one fibrocystic case. Their method of assay consisted of injecting the sample into dogs with permanent pancreatic fistulas, and noting whether a secretory response ensued.

Tables I and II show the distribution of secretin† in the small bowel of 11 controls and 4 fibrocystic disease cases, respectively. For 4 of the controls only one definite assay result could be obtained due to the poor yields of SI or the low sensitivity of some of the assay animals. The comparison of responses of the standard concentrate with the SI from GC (Table II) in an unanesthetized dog with a permanent pancreatic fistula gave a value comparable to the one obtained with the anesthetized acute animal. Secretin occurred in all of the concentrates. Furthermore, the fibrocystic secretin values appear to lie within the normal range.

Conclusions. Secretin was found to occur in the upper bowel of 4 infants and children with cystic fibrosis of the pancreas in amounts comparable to those of controls. The hypothesis that a congenital absence or deficiency of secretin may be an etiological factor of this disease appears to be untenable.

* Senior fellow of the National Research Council; under a grant from the National Foundation of Infantile Paralysis.

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† Cholecystokinin also occurred in the normal as well as in the fibrocystic SI concentrates. Some assay results (units of cholecystokinin per mg of extract; reference 4) are as follows: WS, 2.9; 3.0; ST, 2.9; 2.9; PO (Table II), 0.83; 0.83.

TABLE I.
Occurrence of Secretin in Small Bowel of Controls.

Case	Age	Sex	Pathology	Time interval between death & gut removal, hr	SI yield, mg	Secretin assay (units/mg)	Total secretin unitage
BU*	12 hr	M	Congenital atelectasis	10	22.0	.28; .23	5.7
PO*	24 mo.	F	Encephalitis	19	55.3	<0.2; .08	4.4
WA	2 wk	M	Acute diarrhea	4	13.8	.28	3.9
WI*	9 mo.	M	Acute bronchiolitis	6	41.1	.30; .33	13.
TE	23 mo.	M	Pneumonia; congenital heart disease; Mongolism	28	77.9	.25; .25; .26	19.
EV	7 mo.	M	Amyotonia congenita	29	27.6	.32	8.8
PR*	16 mo.	F	Meningococcus meningitis	11	54.2	.34; .34; .38	19.
WS*	6 yr	F	Leukemia	5	176.	.67; .71; .72	123.
RO	7 yr	M	Acute glomerulonephritis	8	30.3	1.0; 1.4	36.
ST	6 mo.	M	Leukemia	6	82.1	.71; .77; .77	62.
SH	1 mo.	F	Acute diarrhea	20	18.9	.09	1.7

* Negroid.

TABLE II.
Secretin Content of Small Bowel in Cystic Fibrosis of the Pancreas.

Case	Age	Sex	Time interval between death & gut removal, hr	SI yield, mg	Units secretin/mg*	Total secretin unitage
GC	5 mo.	M	3	87.6	.26;* .25	22
CW	5 yr	F	5	188	1.0; 1.05	188
PO	5 yr	F	5	245	.33; .37; .37	88
SB	6 yr	F	11†	253	.40; .40	101

* Assay result obtained with an unanesthetized dog with a permanent pancreatic fistula.
† This bowel specimen, which also contained almost all of the duodenum in addition to three feet of jejunum, was packed in dry ice for a period of 10 hours after autopsy.

The authors are grateful to Miss Elizabeth Perry and Mr. Max Krup for aid in the assays. They acknowledge the cooperation of the pediatrics and pathology services of the University of Illinois, Cook County Hospital, and the University of California.

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Effect of Adrenalectomy on Radiation Induced Mortality of the Mouse.* (17899)

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The relationship of the adrenal-pituitary system to radiation illness has been studied intensely. LeBlond and Segal(1) demonstrated that local destructive irradiation of part of the body resulted in generalized thymo-lymphatic atrophy, which was prevented by adrenalectomy. However, adrenalectomy does not influence the atrophy of lymphoid tissue following direct irradiation. Patt, *et al.*(2,3,4), in a series of studies, dem-

* The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or the endorsement of the Navy Department.

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onstrated a characteristic sequence of events in the adrenals of the irradiated rat. The changes were prevented by hypophysectomy. They concluded that "the radiosensitivity of intact or adrenalectomized mice, with or without exogenous adrenal cortical steroids, is similar". Their conclusions were based on similar mortalities of adrenalectomized and normal mice exposed to one dose of x-ray. This demonstration of the failure of adrenalectomy to increase the mortality of ionizing radiation was inconsistent with past observations on the effect of adrenalectomy on stresses in general. Therefore, we felt it desirable to repeat this work, determining the effect of adrenalectomy on the entire lethal dose curve.

Methods. White Swiss, male, inbred mice, 45 days old were adrenalectomized in a one stage operation through the trans-lumbar route. Combined intraperitoneal sodium pentobarbital and ether inhalation anesthesia was used. Post operatively the mice were given one injection of 0.2 ml of aqueous cortical extract (Wilson) after which they were maintained on Purina Chow and 1% saline. Sham operations in which the adrenals were exposed and replaced were performed by the same methods. The animals were irradiated at the rate of 15 r per minute with the angular beam of a 2.0 Mev GE industrial X-ray tube. The use and properties of this tube have been previously described(5,6). Var-

TABLE I.

Mortality Data for Irradiated Adrenalectomized, Sham Operated, Non-operated Controls and Pooled Mortality Data for 1949. (White Swiss Mice).

Dose X-ray in r	Adrenalectomized 15 days before irradiation			Adrenalectomized 48 hr before irradiation			Sham operated 48 hr before irradiation			Non-operated irradiated controls			Pooled irradiated controls for 1949		
	No. dying	No. exposed	% mortality	No. dying	No. exposed	% mortality	No. dying	No. exposed	% mortality	No. dying	No. exposed	% mortality	No. dying	No. exposed	% mortality
350				1	6	16				0	26	0	0	26	0
400				14	29	48	0	15	0	0	20	0	0	20	0
450	1	6	16.6	5	18	28				1	26	3.8	1	26	4
550	8	18	44.	6	18	33				0	26	0	2	50	8
600				20	28	71	4	15	26	0	20	0	0	20	0
650	12	24	50.	15	26	57				4	26	15	6	62	9
720				6	6	100				10	26	38	10	26	38
750	21	24	87.	11	12	92							300	484	62
775										16	32	50	16	32	50
850	12	12	100.							27	32	84	317	373	85
950	12	12	100.							30	32	90	65	68	96
1050										32	32	100	56	56	100
0	0	12		7	38	18	0	15							

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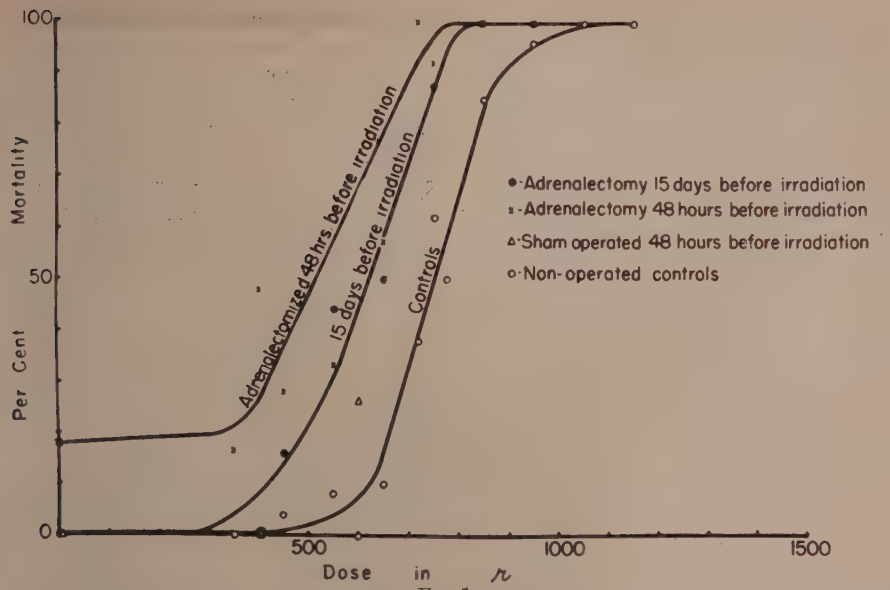

FIG. 1.

28-day dosage mortality curves for adrenalectomized mice, sham operated controls and non-operated controls.

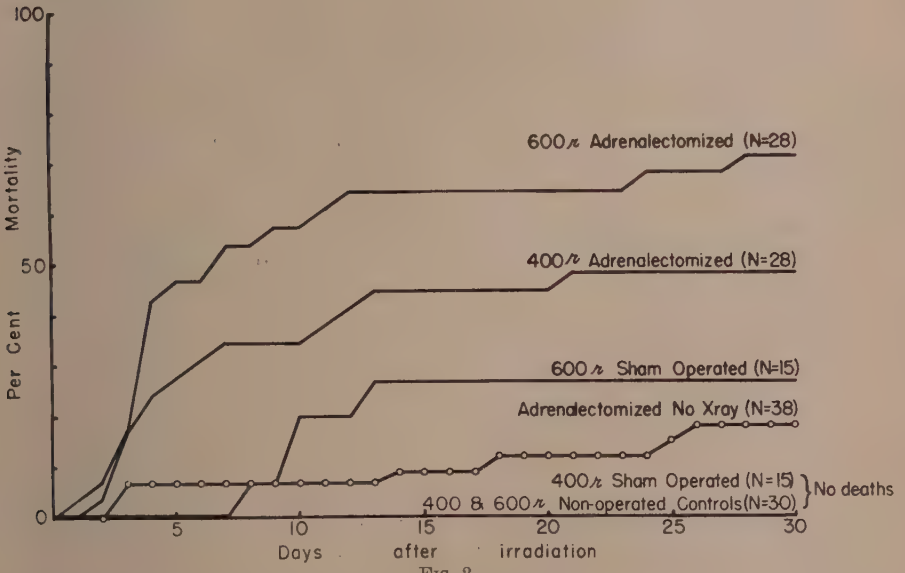

FIG. 2.

Cumulative morality curves of irradiated adrenalectomized mice, sham operated controls, adrenalectomized non-irradiated controls and non-operated controls. For the year 1949 600 r on log-probit analysis corresponded to a mortality of 7%.

ious doses of x-ray were given. The control and operated animals were simultaneously exposed.

Results. Table I and Fig. 1 demonstrate that the mortality of radiation illness is increased by adrenalectomy. The LD_{50} is de-

TABLE II.
Mean Survival Time of Control and Adrenalectomized Mice That Died After Exposure to Various Doses of 2.0 Mev X-ray.
Mean survival time in days ± standard error.

X-ray dose in r	Control mice	Adrenalectomized mice
350	—	8.0 ± .72
400	—	6.8 ± .38
450	—	7.5 ± .41
550	—	7.7 ± .52
600	—	7.3 ± .25
650	10.5 ± .9	7.2 ± .48
720	12.5 ± .5	3.0 ± .68
750	11.0 ± .01	3.5 ± .53
775	13.2 ± .24	—
850	12.8 ± .15	3.2 ± .67
900	10.4	—
950	11.4 ± .06	—
1000	8.8	—
1050	9.8 ± .05	—
1150	6.2 ± .06	—
1920	4.2 ± .04	—
7300	4.5 ± .004	—
9000	4.2 ± .004	—
18000	4.2 ± .007	—
26700	3.7 ± .002	—
64000	0.07	—
128,000	0.07	—

creased by approximately 130 r when mice were adrenalectomized 15 days before irradiation. When mice were irradiated 48 hours after adrenalectomy they seemed even more sensitive to irradiation. It is difficult to properly evaluate this phase of the experiment because of the mortality of the non-irradiated adrenalectomized group. In Fig. 2 the cumulative mortality curves of adrenalectomized, sham operated, and non-operated controls exposed to 400 and 600 r are presented. There were no deaths in the 400 and 600 r non-operated controls and the 400 r sham-operated controls. The mortality of the adrenalectomized animals is significantly greater than that of the sham-operated controls. The mean survival time of mice dying during the 28 day observation period is tabulated in Table II. It is apparent that the survival time of the adrenalectomized mice that died is drastically reduced. In fact, it is roughly comparable to the survival time of mice receiving between 1,000 and 30,000 r. In addition the mice apparently die from "shock" because at autopsy evidence of infection and hemorrhage was rarely seen in the adrenalectomized mice. It will be of considerable interest to see if the histo-pathology and hematology of the irradiated adrenalectomized mouse corresponds to that of normal mice receiving the same or larger amounts of radiation.

All of the adrenalectomized mice that survived irradiation for a period of 28 days were placed on distilled water. None died. Apparently accessory adrenal tissue or fragments had undergone sufficient regeneration to protect the mice significantly against both the effects of irradiation and a low sodium chloride intake. By inference one might say that the adrenalectomized mice would have been even more sensitive to irradiation had a permanent total adrenalectomy been achieved. At autopsy it was not possible to find characteristic adrenal tissue. Bits of tissue from the region of the adrenal vein were sectioned and cells suggestive of adrenal cortical cells were found. Perhaps these mice should be termed "adrenal insufficient" rather than adrenalectomized because of their great capacity to restore adrenal function from fragments or accessory adrenal tissue†.

Conclusions. 1. The adrenalectomized or "adrenal insufficient" mouse is more sensitive to irradiation than the normal mouse. 2. The survival time of adrenalectomized mice is shortened. 3. The cause of death in the adrenalectomized irradiated mouse is apparently due to mechanisms other than infection and hemorrhage.

† Since completion of this work Dr. Henry Kaplan, Department of Radiology, Stanford University School of Medicine, has informed us of technics by which mice can be permanently adrenalectomized and maintained in good health.

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Effect of Sodium Salicylate on Antibodies Produced in Guinea Pigs.* (17900)

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Many investigations have dealt with the effect of salicylates on immunological phenomena. These studies may be divided into 3 groups, namely, the *in vitro* inhibition of antigen-antibody reactions, the *in vivo* inhibition of the production of antibodies, and the *in vivo* inhibition of circulating antibodies. A recent review by Smith(1) dealing with the pharmacology of salicylates makes note of the most important contributions in this field. The only experiment which deals with the *in vivo* inhibition of circulating antibodies by salicylates was carried out by McLennan, Jager and Matson (2) in women whose pregnancy was complicated with erythroblastosis fetalis. Eight to 10 g of acetylsalicylic acid (aspirin) per day for 20 weeks failed to alter the course of the disease. The present experiment was designed to evaluate under more controlled conditions the effect of sodium salicylate in guinea pigs on the antibodies formed in response to the antigens in Rh^+ type O human red blood cells. The immunized animals were divided into 2 groups, one group for the determination of control antibody titers and the other for observation of the effect of injected sodium salicylate on antibody titers.

Methods. Hybrid guinea pigs weighing 250-400 g were used. Under sterile conditions and without anesthesia 2-3 ml of blood was taken from the heart. The syringe contained 1-2 mg of dry heparin. The blood was centrifuged for an hour and 0.65 ml of plasma was used for the antibody assay. The remaining portion was divided for duplicate salicylate determinations. Sterile 3-day-old Rh^+ and Rh^- type O human blood was obtained from the Albert Merrit Billings Hospital Blood Bank. The red blood cells were washed 4 times by centrifuging and resuspending in 0.9% saline under sterile conditions. A hematocrit was done in the usual fashion after bringing the volume of the cells in the suspension to approximately 50%. This preparation of cells was injected intraperitoneally into guinea pigs at 48-hour intervals on the following schedule: initial dose, 1 ml of packed cell equivalent; 2nd dose, 1.5 ml; and 3rd, 4th, 5th, and 6th doses 2 ml. The booster dose given on the 44th day was 2 ml. The method described by Burrows *et al.*(3) was used for determining antibody titers. Two percent suspensions of washed Rh^+ or Rh^- type O cells were used as the antigen in the antibody assay. Plasma salicylate concentration was determined by the method of Brodie *et al.*(4). It was found in preliminary experiments that normal guinea pig plasma did not interfere with this colorimetric determination of salicylate and that after the highest dosage used in these experiments no salicylate could be detected 12 hours after injection. The dosage schedule for the salicylated animals is given in the accompanying table.

Results and discussion. The data are summarized in the table. Before immunization the average blood plasma Rh^+ and Rh^- titer was respectively, 1:38 and 1:30. The Student t test(5) indicated that the average Rh^+ titer did not differ from the average Rh^- titer. A p value of 0.05 or less is considered significant. Eight Rh^+ and 7 Rh^- antibody assays were done at various times after immunization on the plasma of non-

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TABLE I. Rh⁺ and Rh⁻ Antibody Titers to Human Rh⁺ Type O Red Blood Cells in Guinea Pigs With and Without Injected Sodium Salicylate.

Days after immunization	No. of animals		Control plasma antibody titer				Salicylated plasma antibody titer				Avg salicylate plasma conc.,† mg %	Sodium salicylate dosage schedule,‡ (g/kg)
	Control	Salicylated	Avg Rh⁺ titer	S.D.*	Avg Rh⁻ titer	S.D.	Avg Rh⁺ titer	S.D.	Avg Rh⁻ titer	S.D.		
Before	55	—	1:38	1:41	1:30	1:32	—	—	—	—	—	—
4	16	3	1:778	1:477	1:819	1:439	1:300	1:110	1:300	1:110	40. (29-45)	.32 q.4h.
12	12	2	1:312	1:340	1:136	1:539	1:50	—	1:20	—	15. (10-21)	.16 q.3h.
20	5	9	1:38	1:16	1:38	1:16	1:42	1:50	1:46	1:42	15.5 (6-28)	.16 q.3h.
28	6	6	1:50	—	1:50	—	1:50	—	1:50	—	15.2	.16 q.3h.
36	5	8	1:50	—	1:50	—	1:53	1:35	1:53	1:35	37. (28-42)	.3 q.4h.
44	13	—	1:69	1:48	1:65	1:29	—	—	—	—	—	—
44						Booster						
48	3	6	1:1500	1:866	1:1500	1:866	1:1750	1:612	1:1500	1:548	49.6	.24 q.3h.
74	6	—	1:5333	1:2479	—	—	—	—	—	—	—	—

* Stand. dev. $\sqrt{\frac{\Sigma d^2}{n-1}}$.

† 3 hours after the last injection of sodium salicylate.

‡ Given intraperitoneally during the 24 hr immediately preceding the withdrawal of blood samples.

salicylated immunized guinea pigs. Although antibodies were formed against the Rh⁺ type O human red blood cells, none of the antibody assays done showed significant antibody formation against the Rh⁺ antigen. Hence, the data indicated that the Rh⁺ antigen added little to the antigenicity of the type O cells. On the 12h day after completion of immunization, plasmas from 7 different guinea pigs were incubated for 2 hours with Rh⁻ type O cells in order to absorb the type O antibody. Antibody titers were determined on these plasma preparations; three of the 7 plasmas showed a partial but definite agglutination of Rh⁺ cells.

The average Rh⁺ and Rh⁻ circulating antibody level rose on the 4th day after immunization to 1:778 and 1:819, respectively, fell to 1:312 and 1:136, respectively, on the 12th day and by the 20th day had fallen to the baseline level. The Student t test was used to compare the average Rh⁺ and Rh⁻ antibody titers before immunization with the average Rh⁺ and Rh⁻ antibody titers of the 20th, 28th, 36th, and 44th day. No significant difference was noted. On the 44th day a booster injection of Rh⁺ type O cells was given. Four days after the booster injection the average Rh⁺ and Rh⁻ titer rose to 1:1500. The average Rh⁺ titer was 1:5333 30 days after the booster dose. The results of the immunization with Rh⁺ type O cells indicate then, that antibodies are formed to the antigens in the Rh⁺ type O cells. These circulating antibodies disappear by the 20th day after immunization. A booster dose 44 days after initial immunization produces a greater and more lasting circulating antibody titer.

On the 3rd, 11th, 19th, 27th, 35th, and 47th day following immunization several of the guinea pigs were given different doses of sodium salicylate and bled 3 hours after the last injection. On the 4th day after immunization the average salicylated plasma Rh⁺ and Rh⁻ titers were approximately 37% of the control. The student t test indicated that the average salicylated plasma Rh⁺ and Rh⁻ titers on the 4th day after immunization differed significantly from the average control Rh⁺ and Rh⁻ titers. On the 12th day after immunization the average salicylated plasma

Rh^+ and Rh^- titers were approximately 15% of the control, a significant difference. The average Rh^+ and Rh^- titers on the 20th, 28th, and 36th days, which did not differ from the baseline anti-human hemoagglutinins were not depressed by sodium salicylate. This was not an unexpected finding. On the 48th day after immunization or 4 days after the booster injection it was found that the average Rh^+ or Rh^- titer was not depressed by salicylate. These data on the 48th day after immunization are of interest in view of the findings on the 4th and 12th day after immunization. The average plasma salicylate concentration on the 4th day after immunization was 40 mg %; whereas the plasma salicylate level on the 48th day after immunization was 50 mg %, an increase of 25%. A plasma salicylate level of 50 mg % is toxic. The average Rh^+ and Rh^- titers on the 4th day after immunization were 1:778 and 1:819, respectively; whereas the average Rh^+ and Rh^- titers on the 48th day after immunization were 1:1500, an increase of approximately 93% and 83%, respectively. Comparing the average plasma salicylate levels and the average plasma antibody titers on the 12th and 48th day after immunization, it was found that plasma salicylate level on the 48th day was 236% higher and the Rh^+ and Rh^- titers were 381% and 1000% higher, respectively. Since the increase in plasma salicylate concentration was not equal to the increase in the antibody titer on the 48th day as compared to the 4th and 12th day, it is postulated that there was not enough salicylate present to inhibit the antibodies on the 48th day. This theory is supported by several facts. In the range of Rh^+ or Rh^- titers between 1:136 and 1:819 the antibody titer was depressed by non-toxic plasma concentrations of salicylates. However, if the antibody titer was 1:1500, it was not depressed even by toxic plasma concentrations of salicylates. The *in vitro* inhibition of antibodies has been shown to be proportional to the concentration of salicylates(6,7).

Summary. Antibody titers produced in guinea pigs receiving Rh^+ type O human red blood cells intraperitoneally were compared each week after immunization with those produced in similarly treated guinea pigs which also received varying doses of sodium salicylate for one day before drawing blood for the measurement of antibody titers and salicylate plasma levels. Rh^+ type O human red blood cells were antigenic in the guinea pig. The Rh^+ antigen added little to the antigenicity of the type O cells. The average Rh^+ or Rh^- antibody titers rose to 1:778 or 1:819 on the 4th day after immunization, fell to 1:313 or 1:136 on the 12th day and by the 20th day had fallen to 1:50, the baseline level. A more lasting circulating antibody titer was produced after a booster dose of Rh^+ type O cells. It is concluded that the natural anti-human hemoagglutinins cannot be depressed by sodium salicylate. The data indicated that the depressant action of sodium salicylate on the Rh^+ type O human antigen-antibody reaction in guinea pigs was limited by the toxicity of salicylates. Rh^+ or Rh^- titers between 1:136 and 1:819 were depressed significantly by non-toxic plasma levels of sodium salicylate (15-40 mg %); whereas Rh^+ or Rh^- titers of 1:1500 were not depressed even by toxic plasma levels (approximately 50 mg %).

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Use of Antitryptic Agents in Tissue Culture. II. Egg-White Antitrypsin.*† (17901)

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An earlier communication(1) from this laboratory reported studies on the use of crude soybean trypsin-inhibitor as a means of preventing the digestion of plasma and fibrin clots that frequently occurs during the growth of cell colonies *in vitro*. This substance, though effective as an antiproteolytic agent, was found to inhibit the growth of the cultures and to prolong the coagulation time of the plasma. These results agreed closely with those obtained by Fischer(2), who employed crystalline soybean antitrypsin. Since the toxicity of the soybean factor appeared to limit its usefulness in tissue culture, an investigation was made of ovomucoid(3), the antitryptic substance in egg white.

Methods. Egg-white antitrypsin was prepared from freshly-laid hens' eggs by the trichloracetic acid and acetone method of Lineweaver and Murray(3). Other preparations were made by a salt-fractionation procedure(3) and by the ammonia and alcohol method of Balls and Swenson(4). In certain experiments, untreated egg yolk, untreated egg white and lyophilized egg white were employed. The activity of each preparation was determined by its ability to prevent the digestion of casein by crystalline trypsin, according to the method of Kunitz(5). These tests were carried out as described for the experiments with soybean antitrypsin(1). All solutions were sterilized by passage through UF fritted glass filters (Corning) and were stored in the refrigerator. Culture strains of fibroblast-like mesenchyme cells were derived from the leg muscle of 11-day chick embryos and were cultivated in D-3.5 flasks through at least 7 to 10 passages (weeks) before use. The culture medium was comprised of chicken plasma, a balanced salt mixture(6) and chick embryo extract. These ingredients, the manner in which they were combined, and the actual culture procedures have already been described(1). In all experiments, an additional layer of the plasma-saline-extract mixture was added to half of the control cultures to minimize digestion, and in certain experiments *all* cultures received the extra layer. This procedure, sometimes referred to as "patching," permitted culture growth without noticeable digestion, so that any growth-inhibiting effects of the antitrypsin could be detected.

Experimental. Egg-white antitrypsin, prepared by the trichloracetic acid and acetone method, was added to several series of sister cultures at final concentrations of 5.0, 2.5, 1.0, 0.5, 0.25 and 0.1 mg per ml. These cultures, together with their controls (without antitrypsin) were incubated at 38° and were observed each day for signs of digestion. It was found that all cultures containing egg-white antitrypsin completely digested the plasma coagulum within 3 days. It was also found that digestion occurred in the presence of antitrypsin even though, in some instances, the control cultures, with and without an additional plasma layer, did not digest the coagulum (Fig. 1-4). The presence of high concentrations of antitrypsin (5.0 to 2.5 mg per ml) caused a cloudiness in the clot, but this effect was not apparent at lower levels.

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† Grateful acknowledgement is made to Mrs. C. J. Porter and Mr. C. J. MacFayden for technical assistance.

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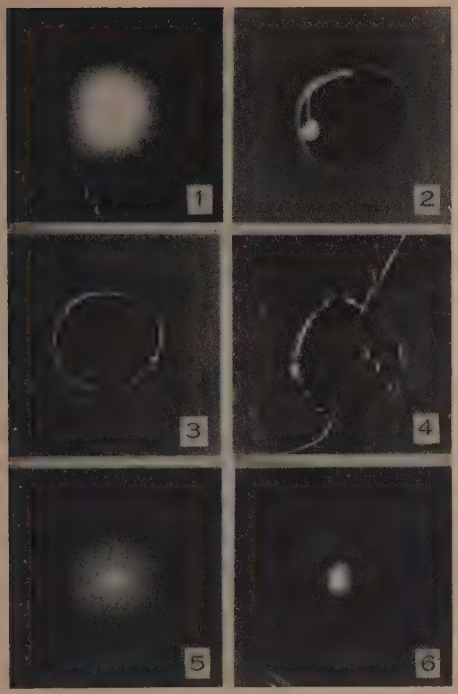

FIG. 1, 2, 3, and 4.
Seven-day sister cultures of 7th passage chick fibroblasts cultivated in a plasma coagulum in the absence of antitrypsin (Fig. 1), in the presence of 5.0 mg egg-white antitrypsin per ml (Fig. 2), 2.5 mg per ml (Fig. 3), and 1.0 mg per ml (Fig. 4). × 2.

FIG. 5 and 6.
Seven-day sister cultures of 16th passage chick fibroblasts cultivated in a plasma coagulum in the absence of antitrypsin (Fig. 5), and in presence of 0.5 mg egg-white antitrypsin per ml (Fig. 6). × 2.

Similar results were obtained with material prepared by salt fractionation and by the ammonia and alcohol procedure. It was concluded, therefore, that egg-white antitrypsin stimulated proteolytic activity instead of preventing it.

The presence of egg-white antitrypsin, even in low concentrations, was found to inhibit, very appreciably, the growth of the cultures. This inhibition was most apparent with antitrypsin prepared by the ammonia and alcohol method (Fig. 5 and 6) but was found to occur to a lesser degree with all antitryptic preparations tested. Comparative experiments were carried out in which fresh egg yolk, fresh egg white and lyophilized egg white were incorporated in the culture medium. The presence of small amounts of egg yolk (0.01 to 0.001 ml) was somewhat inhibitory to the growth of the cultures, whereas larger amounts suppressed growth almost completely. Both raw and lyophilized egg white were inhibitory to growth and did not protect the plasma clots from digestion.

None of the antitryptic preparations tested was observed to prolong the coagulation time of the plasma. In this respect egg-white antitrypsin differs from soybean antitrypsin (1).

In view of the failure of egg-white antitrypsin to prevent plasma digestion by tissues cultivated *in vitro*, test-tube experiments were made to study its effect upon the hydrolysis of casein and fibrin by crystalline trypsin. The activity of the various egg-white preparations was found to correspond closely with that reported by Lineweaver and Murray(3), and to represent roughly an 8-fold increase in potency over that of lyophilized egg white. The antitryptic activity was exerted equally well regardless of whether fibrin or casein was employed as the substrate. From these results, it was concluded that the preparations found to be ineffective in tissue cultures were fully potent in their ability to prevent the proteolytic action of crystalline trypsin. It was also observed that the digestion of casein and of fibrin was increased by the presence of very small amounts of egg-white antitrypsin. This effect was exerted by concentrations considerably below the levels found to be effective in preventing the action of the standard amount of trypsin (10 μg).

Discussion. Early tissue culture workers attempted to solve the troublesome problem of plasma digestion by the addition of various protective substances. Carrel and Ebeling(7) incorporated 0.25% egg-yolk suspension in their media; and Carrel(8) reported the use of serum, sodium linoleate and egg-yolk suspension to prevent clot lysis. These attempts were not particularly successful,

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however, and complete digestion of the coagulum is now commonly avoided by the successive addition of fresh layers of plasma, as they become necessary. Since this procedure is laborious and requires large amounts of plasma, it was hoped that the problem might be solved by the use of antitryptic agents. The present experiments, involving more than 150 cultures, would seem to eliminate egg-white antitrypsin as a possible antiproteolytic agent for use in tissue cultures.

The failure of egg-white antitrypsin to prevent plasma digestion in tissue cultures is in marked contrast to its ability to prevent the proteolytic action of trypsin upon either casein or fibrin substrates. However, soybean trypsin-inhibitor has been found effective both in tissue culture and in test-tube experiments(1). These divergent results may possibly be attributed to a difference in the degree of specificity of the two antiproteolytic agents. Egg-white antitrypsin has been shown(9,10) to be almost entirely specific for trypsin, whereas soybean antitrypsin has been found to be a general inhibitor of proteolytic enzymes(10). The general antiproteolytic activity of the soybean trypsin-inhibitor might account for its interference with normal plasma coagulation, whereas egg-white antitrypsin, which is active only against trypsin, does not interfere.

Digestion of the plasma coagulum by cells cultivated *in vitro* has been attributed to the secretion of proteolytic enzymes(8,11) or to the elaboration of a substance that activates profibrinolysin to fibrinolysin(12,13). The present findings do not furnish evidence in support of either of these hypotheses, but they do render it unlikely that plasma digestion is caused by *trypsin*.

Summary. Egg-white antitrypsin not only fails to prevent but actually stimulates the digestion of plasma by cells cultivated *in vitro*. The presence of even low concentrations of antitrypsin inhibits tissue-culture growth but does not interfere with normal plasma coagulation. The antitrypsin preparations shown to be ineffective in tissue cultures strongly inhibit the proteolytic activity of crystalline trypsin in test-tube experiments.

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Antibacterial Activity of Hydrolyzed Red Blood Cells *in Vitro*.* (17902)

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In our recent studies(1) the presence of an antibacterial factor in ticks was interpreted mainly as a biological phenomenon which may have bearing on the natural transmission of arthropod-borne infections. The higher antibacterial potency of blood engorged ticks as compared with unfed specimens was a significant indication of a possible growth inhibitory role of blood *per se*. Preliminary tests gave evidence that the tryptic digest of guinea pig blood exhibits a marked growth inhibition of *Bacillus subtilis*(1). This observation stimulated the present inquiry as to whether the blood of various animals may serve as a source of antibacterial substances. The possible relationship of the following antibacterial substances of animal origin have been considered in the present study: Flem-

* Aided by a grant of Lilly Research Laboratories.

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ing's lysozyme; the antibacterial peptides isolated by Bloom, Watson, *et al.*(2) from the cells of various animal tissues; bacteriostatic extracts from rabbit liver and human placenta reported by Konikova, *et al.*(3); and "erythrin" originating from red blood cells and quoted by Waksman(4).

Recent developments in the field of antibiotics of animal origin are reflected by the series of papers of Pavan(5) in Italy. Extracts from tissues of helminths, arthropods, molluscs, fish, amphibia and reptiles were shown by this author to possess antibacterial properties.

Material and methods. Blood samples from man, cattle, dog, rabbit and chicken were allowed to coagulate, the clot separated, triturated in distilled water and lyophilized. The desiccated material (0.1 g) was suspended in 5.0 ml of 0.1 M phosphate buffer (pH 7.8) containing 0.1 ml of 5% trypsin (Pfanstiel 1:110) and the mixture incubated for 5 hours at 37°C. This was followed by heating in 65°C waterbath for 30 min. The clear, dark-brown tryptic digest was tested for antibacterial activity by occasional assays with streak plates, but mainly by the spot dilution method on tryptone glucose agar plates previously flooded with bacterial cultures. This technic, previously described(1) for antimicrobial assays, proved very convenient, serving the double purpose of qualitative and quantitative evaluation. In the latter case, the gradual diminishing diameter of the circular inhibition zones in serial dilutions of the material placed in a standard drop (gauge 27) on the flooded plate can be measured and compared with circular spots produced by a conventional antibiotic of known potency. The same technic as applied to blood was used later for the hydrolysis of bovine hemoglobin powder (Armour Laboratories). The following modification was also used: before adding trypsin hemoglobin was denatured by heating at 65°C for 30 min in N/10 NaOH followed by adjustment to pH 7.8 with N HCl.

FIG. 1.
Growth inhibition of *Bacillus subtilis* as shown by interrupted streaks on agar plate in areas of contact with trypsin digested human red blood cells (dilution 1:50).

Experiments and results. A complete growth inhibition of *Bacillus subtilis* on streak agar plates was shown in contact areas with processed blood of human, cattle, dog and rabbit origin (Fig. 1) corroborating the previously observed phenomenon with guinea pig blood(1). Since this observation was made with washed and digested red cells, it was concluded that red cells or hemoglobin liberate the active principle inasmuch as lyophilized serum and washed, dried fibrin (Armour) after hydrolysis showed no action against *B. subtilis*. Crude extracts of blood of man, cattle, dog and chicken were tested against a series of gram-positive and gram-negative bacteria. The qualitative tests with mammalian blood indicated a general uniformity in the degree of growth inhibition (Table I) as compared with the lower activity of chicken blood. Of the three mammals tested, cattle blood showed the greatest activity. The highest sensitivity was shown by the spore-bearing bacilli, *Sarcina lutea* and *Staphylococcus aureus*, but inhibition of growth was also recorded among some gram-negative organisms (Table I).

2. Bloom, W. L., Watson, D. W., Cromartie, W. J. and Freed, M. *Jour. Inf. Dis.*, 1947, v80, 41.

3. Konikova, A. S., Urasova, A. P. and Asarkh, *Acad. Sc. U.S.S.R.*, 1945, v47, 565.

4. Waksman, S. A., Microbial antagonists and antibiotic substances, N. Y., p. 415, 1947.

5. Pavan, M., *La Ricerca Scient.*, 1949, v19, 1011.

TABLE I.

Antibacterial Activity *in Vitro* of Hydrolyzed Blood as Established by Complete + or Partial ± Inhibition of Growth.

Spot dilution method on flooded agar plates. All dilutions 1:50 containing 20 mg of the raw dehydrated material per 1.0 ml or 0.4 mg per drop.

Organism	Blood origin			
	Human	Cattle	Dog	Chicken
Staphylococcus albus	±	+	±	±
" *aureus*	+	+	+	—
Sarcina lutea	+	+	+	—
Bacillus subtilis	+	+	+	+
" *anthracis*	+	+	+	—
" *pumilus*	+	+	+	±
" *megatherium*	+	+	+	+
Salmonella typhimurium	±	+	±	±
Escherichia coli	±	+	±	+

Thus, by enzymatic hydrolysis of red blood cells substances were liberated which exerted an inhibitory action *in vitro* on various organisms. The degree of growth inhibition of *B. subtilis* as a test organism was estimated in serial dilutions noting the endpoint of activity and measuring the diameter of the circular zones produced by the active material. Under these constant conditions the radius of activity varied somewhat with the blood donor. For example, the average diameter for zones produced by human blood was approximately equal to 17-19 mm, for cattle blood 15-18 mm, for dog blood 14-16 mm, whereas the range for chicken blood was from 11 to 15 mm, all measurements being taken at the same initial dilution of 1:100 (Fig. 2). Thus it became evident that the antibacterial activity of human and cattle blood cells was relatively higher than that of dog or chicken, not only in the size of the inhibition zones, but also by the endpoint titers in serial dilutions.

Attempts to localize the source of the antibacterial products of blood gave evidence that the tryptic digest of hemoglobin powder is at least equally as inhibitory to *B. subtilis* as the blood products. This was followed by quantitative evaluation on a wide spectrum of various organisms (Table II). Whereas *B. subtilis* and other spore-bearing bacilli show the highest sensitivity, the inhibitory effect on gram-positive cocci and on some gram-negative bacilli is of interest. In this regard, the considerable sensitivity of *Streptococcus viridans* may prove of practical importance. It may be noted that the usual resistance of *Pseudomonas aeruginosa* to antibiotics is lowered by the hemoglobin hydrolysates. For reasons of comparison with conventional antibiotics, Bacitracin was chosen in view of its similar spectrum of activity. Using the spot dilution technic with *B. subtilis* circular zones of 15 mm diameter were produced by a standard drop of Bacitracin in a concentration of 10 mg/ml. This

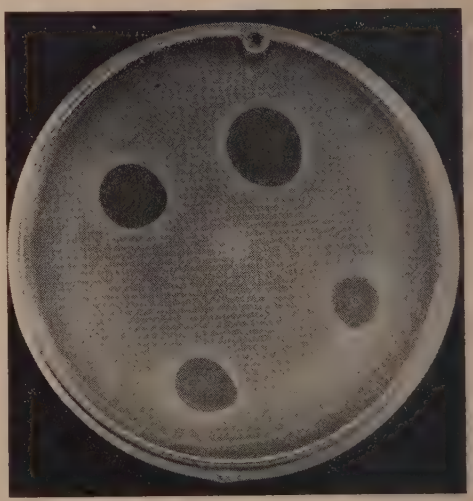

FIG. 2.

Growth inhibition of *Bacillus subtilis* on flooded agar plate as shown by circular zones produced by digested rabbit blood cells. Spot dilution method beginning with 1:100 in the largest circle and ending with 1:1,600 in the smallest.

TABLE II.
Antibacterial Activity *in Vitro* of Hydrolyzed Bovine Hemoglobin.
Spot dilution method on flooded agar plates.

Test organism	Endpoint		Inhibition
	mg/ml	Dilution	
Staphylococcus albus	2.5	1:400	Complete
" *aureus*	10.0	1:100	"
Sarcina lutea	10.0	1:100	"
Streptococcus viridans	1.25	1:800	"
Bacillus subtilis	0.12	1:9600	"
" *anthracis*	0.24	1:4800	"
" *pumilus*	1.25	1:800	"
" *megatherium*	0.24	1:4800	"
Salmonella oranienburg	10.0	1:100	"
Escherichia coli	2.5	1:400	"
Pseudomonas aeruginosa	2.5	1:400	"

diameter corresponded to that produced by an identical drop of bovine hemoglobin hydrolysate in a concentration of 0.2 mg/ml. In other words, the potency of our product in respect to *B. subtilis* compares well with that of a recognized antibiotic. However, this comparison of a crude product with a purified antibiotic is only of relative value.

Initial toxicity tests showed that daily intra-abdominal injection of 20 mg of the blood hydrolysate per guinea pig (450-600 g) over a period of 5 days had no untoward effect. Similar tests on mice inoculated intra-abdominally with 1.0 ml of the crude hydrolysate containing 20 mg of the dry material showed that these animals were unaffected after a total of 160 mg were administered over 8 days.

The physical properties of the antibacterial substance or substances derived from red blood cells are characterized by a significant thermostability resisting exposure to 121°C for 15 minutes in a liquid medium at a pH range from 7.8 to 9.0. In contrast to the unchanged appearance of the autoclaved tryptic digest within this pH range accompanied by a moderate loss in antibacterial activities against *B. subtilis,* autoclaving of the blood hydrolysates at pH 5.5 or lower causes a heavy precipitate and complete loss of antibacterial activities of the supernate. Similar properties were shown by extracts of blood engorged ticks as reported in our recent study (1). The activity of the clear, dark brown liquid of blood hydrolysates indicates solubility of the active principle in aqueous solutions of phosphate buffer or in saline, with optimum activity in the range of pH 7.0 to 8.0.

Discussion. The continuity in the transmission of infectious agents in nature by biological vectors, the bacterial sterility of the blood sucking arthropods, their remarkable resistance to specific pathogenic agents, and above all, the observations on the bacteriostatic factors in blood engorged ticks, have prompted the present study. Of these phenomena, the resistance or the so-called natural immunity of the arthropod vector to organisms pathogenic to higher animals dominates the host-parasite problem. The survival of the resistant host may be due to previous contacts with the pathogenic agent or perhaps to the production of metabolic substances, some of which may neutralize or suppress the activities of the parasite. In this regard, the isolation of an anthracidal polypeptide from leukocytes and other animal cells by Watson, Cromartie, *et al.*(6) indicates that such substances as antibacterial peptides of animal origin may be vital contributors to host resistance. Another and earlier example is Fleming's lysozyme as a typical representative of an antimicrobial substance of animal origin. In view of its wide prevalence in animal tissues, lysozyme and its possible relationship to our products was considered.

For this purpose, crystalline egg white

6. Watson, D. W., Cromartie, W. J., Bloom, W. L. *et al., J. Inf. Dis.,* 1947, v80, 121.

lysozyme (Armour Company) was submitted to conditions under which blood hydrolysates exhibit their optimal antibacterial activities with the result that contrary to the pH range of 7.8 to 9.0 in which our products resist autoclaving, lysozyme activities were destroyed regardless of pH (5.4-9.0). Furthermore, the stability of lysozyme in acid solution and its inactivation in alkaline solutions is contrary to the characteristics of our product. Although the specific identity of our antibacterial substance or substances has not been determined as yet, it is plausible that the ultimate products obtained from hemoproteins by hydrolysis are peptides or amino acids. In its present crude form, the material is most probably a hydrolysate complex rather than an entity. As to the antibacterial substances of animal origin reported by others and previously mentioned by us, the bacteriostatic preparation from liver obtained by Konikova *et al.*(3) by acid precipitation is water-insoluble and apparently differs from our product inasmuch as they obtained no bacteriostatic fractions from processed blood. A sample of "erythrin" made available to us by Dr. Waksman was highly acid and insoluble in water.

It becomes apparent from the present study that the bacterial growth inhibition *in vitro* compares with the phenomenon induced by antibiotics. This similarity refers particularly to the selective antibacterial action of the substances released from red blood cells, affecting primarily the gram-positive organisms. Considering this selectivity and the biological source of origin of our products, their inclusion into a broader concept of antibiotics seems to be justified. This would be also in line with the recognition of antibiotic products from higher plants(7).

Summary. Red blood cells of various animals were submitted to enzymatic hydrolysis in alkaline medium, the resulting products exhibiting *in vitro* marked antibacterial activities. The latter covered a relatively wide spectrum of most gram-positive and a few gram-negative organisms. The highest activity was displayed by hydrolysates of human and bovine hemoproteins. Hydrolyzed bovine hemoglobin powder likewise exhibited antibacterial properties. These antibacterial products are heat-resistant and water-soluble. Their optimum activity lies in the pH range of 7.0 to 8.0. It is assumed that the active principle of the crude substance is a peptide-amino acid complex. This complex proved to be nontoxic to guinea pigs and albino mice. Its relation to other antibiotics is discussed.

7. Symposium on Antibiotics. *J. Clin. Invest.*, 1949, v28, 894.

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Antigenic Identity of *Shigella alkalescens* Type I and Kauffmann's Escherichia O Group 1. (17903)

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Antigenic relationships between the coliform bacteria and members of the genus Shigella have been studied by many workers (1-5). The sharing of minor antigenic components by members of the two groups has been noted frequently, but complete antigenic identity has been demonstrated in only a few instances. The closest relationships to the coliform group have been found in *Shig-*

1. Carpenter, P. L., and Stuart, C. A., *J. Immunol.*, 1950, v64, 237.

2. Felsenfeld, O., and Young, V. M., *Am. J. Dig. Dis.*, 1945, v12, 396.

3. Ferguson, W. W., and Wheeler, W. E., *J. Bact.*, 1946, v51, 107.

4. Kauffmann, F., *Acta Path. et Microbiol. Scand.*, 1944, v21, 72.

5. Wheeler, K. M., Stuart, C. A., and Ewing, W. H., *J. Bact.*, 1946, v51, 169.

ella alkalescens and *Sh. dispar,* both of which are generally considered nonpathogenic. Stuart and his co-workers(6) described a series of strains which included typical *Escherichia coli,* anaerogenic and slow lactose-fermenting paracolons, and biochemically typical *Sh. alkalescens,* all with identical O antigens. Ewing(7) has described a similar biochemically intergrading series between *E. coli* and *Sh. dispar,* with indistinguishable O antigens. The systematic analysis of antigens shared by *E. coli* and other bacteria has been handicapped by the serological complexity of Escherichia, and the lack of a formal classification of the antigens of this genus. Such a classification has recently been compiled by Kauffmann(8). Strains representing his O groups 1 through 110 were sent by Dr. Kauffmann to this laboratory, and form the basis of the present study.

Methods and results. Only the heat-stable O antigens were studied in the present experiment. All antigens were prepared from 18 hour extract broth or agar cultures, and heated in flowing steam for one hour. Agglutination tests were incubated at 50°C for 18 hours. Antigens of type strains of all the Kauffmann O groups 1 through 110 were tested for agglutination in a 1:100 dilution of *Sh. alkalescens* Type 1 antiserum with a titer of 1:1600. The only strains which gave more than partial agglutination were those of O groups 1 and 31. Further tests showed that *Sh. alkalescens,* O 1 and O 31 were agglutinated to the same titers in antiserums prepared from each of the 3 strains. Agglutinin absorption tests were performed, and resulted in complete reciprocal absorption between *Sh. alkalescens* and O 1, while absorption of the same serums with O 31 failed to lower the titer for either of the other organisms.

Fifteen locally isolated strains of *E. coli* previously identified as belonging to O group 1 on the basis of direct agglutination, and ability to agglutinate in O 1 antiserum absorbed with the O 31 type strain, and nine strains of *Sh. alkalescens* were included in the study. Among the local strains was a paracolon isolated from the feces of a normal person and biochemically closely related to *Sh. alkalescens.* The paracolon, like *Sh. alkalescens,* fermented glucose, maltose, mannitol, xylose, arabinose, glycerol, rhamnose, and sorbitol with acid but no gas, produced indol, failed to ferment sucrose and salicin, to liquefy gelatin, grow in Koser's citrate medium, or to produce H_2S or acetylmethylcarbinol. It differed from *Sh. alkalescens* in producing acid from lactose after 6 days' incubation, in giving delayed but permanent acidity in milk, failing to ferment dulcitol and being slightly motile. This organism, together with a biochemically typical *E. coli* belonging also to O group 1, was present in large numbers in the feces of this individual over a period of several months. All of the strains tested were agglutinated to approximately the same titer by 3 *E. coli* O group 1 antiserums and by 3 *Sh. alkalescens* antiserums. Each of the coli antiserums was absorbed with several different strains of *Sh. alkalescens* and the alkalescens antiserums with several strains of *E. coli.* In all cases complete absorption of agglutinins was obtained. A representative series of results is shown in Table I. Results with other strains were similar to the ones used as examples.

The Kauffmann type strains were also tested for agglutination in single dilutions of antiserums of *Sh. dysenteriae,* ambigua, sonnei, and all the Flexner and Boyd types of *Sh. paradysenteriae.* Though minor relationships were found several times, the Escherichia strains were rarely agglutinated to more than a fraction of the titer of the serums when titrations were done. In 2 cases, however, very close relationships were found. Preliminary experiments suggest that Kauffmann's O group 53 and *Sh. boydii* IV (P274), and O group 79 and *Sh. boydii* V (P143), may be antigenically identical, but the number of strains tested was too small to warrant definite conclusions.

Discussion. So far as can be determined, a correlation between the common antigens

6. Stuart, C. A., Rustigian, R., Zimmerman, A., and Corrigan, F. V., *J. Immunol.,* 1943, v47, 425.
7. Ewing, W. H., *J. Bact.,* 1949, v58, 497.
8. Kauffmann, F., *J. Immunol.,* 1947, v57, 71.

TABLE I.
Agglutination Titers of Representative Strains of *Shigella alkalescens* and *Escherichia coli* in Unabsorbed and Absorbed Serums of Both Species.

Antiserum	Absorbed by	O antigens: *Escherichia coli* K1	*Escherichia coli* SS1	*Escherichia coli* Paracolon	*Shigella alkalescens* Fong	*Shigella alkalescens* Wagner	*Shigella alkalescens* Gordon
		E. coli					
K1*	—	12,800	12,800	6400	6400	6400	6400
,,	Fong	0†	0	0	0	0	0
,,	Wagner	0	0	0	0	0	0
SS1	—	6400	6400	6400	3200	3200	3200
,,	Fong	0	0	0	0	0	0
,,	Wagner	0	0	0	0	0	0
Paracolon 1	—	3200	3200	3200	3200	3200	3200
,,	Fong	0	0	0	0	0	0
,,	Wagner	0	0	0	0	0	0
		Sh. alkalescens					
Fong	—	3200	3200	1600	1600	1600	1600
,,	K1	0	0	0	0	0	0
,,	SS1	0	0	0	0	0	0
,,	Para. 1	0	0	0	0	0	0
Wagner	—	6400	6400	6400	3200	3200	3200
,,	K1	0	0	0	0	0	0
,,	SS1	0	0	0	0	0	0

* Kauffmann's strain U5/41.
† Negative at 1:40 dilution.

of *Sh. alkalescens* and certain coliform and paracolon strains has not previously been made in relation to Kauffmann's Escherichia O groups. The present experiments indicate the O antigens of Kauffmann's O group 1 and those of *Sh. alkalescens* are identical. Kauffmann (8) named group 1 among the 8 groups most frequently isolated by him from pathological materials. Strains belonging to this serologic group have been isolated many times in this laboratory, both from feces and from urine. Since they are apparently widely distributed, it is not surprising that they should occur fairly frequently together with *Sh. alkalescens*, though the marked biochemical differences between typical strains of *E. coli* and *Sh. alkalescens* make it unlikely that the two would be mistaken for each other. Knipschildt, as quoted by Kauffmann (8), has found that paracolon strains of varying biochemical reactions can be classified in the O groups as frequently as can true Escherichias, and the experience in this laboratory has been the same. Of particular interest is the occurrence of anaerogenic, slow lactose-fermenting members of the group, like the organism described here. The possibility of confusion between such strains and *Sh. alkalescens* is very great, since careful tests for motility and prolonged incubation of carbohydrate media may be necessary for differentiation. The motile, lactose-fermenting or gas-producing strains reported in the literature as *Sh. alkalescens* on the basis of their antigenic characters are presumably paracolons belonging to O group 1.

Summary. Agglutination and agglutinin absorption tests performed with fifteen *E. coli* strains of Kauffmann's O group 1 and nine strains of *Sh. alkalescens* demonstrated that their O antigens were indistinguishable.

Since this manuscript was submitted for publication an article by E. Frantzen has appeared in *Acta Path. et Microbiol. Scand.*, 1950, v27, 236. This article comes to the same conclusion as the present one concerning the antigenic identity of *Shigella alkalescens* Type I and Kauffmann's coli O group 1.

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Changes of the Ta Wave in Standard Leads Following Stimulation of the Vagus Nerves.* (17904)

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In experiments in which the right or left vagus nerves are stimulated in the neck with a faradic current during regular sinus rhythm marked changes of the P-R segments develop in the standard leads. The experiments were performed on dogs which were anesthetized with nembutal. The heart was exposed by removal of the sternum and the adjacent parts of the ribs and by opening the pericardium. The vagus nerves were severed in the neck and the peripheral end was stimulated with the aid of a Cambridge Inductorium.

Results. Fig. 1a shows a sinus tachycardia with a rate of 192. The P waves are large (lead II). The P-R segment is within the zero line. Following stimulation of the right vagus nerve, for 2.2 seconds, the first P wave differs in form from the others; the P-R segment is markedly depressed. This depression persists in a number of subsequent P waves which resemble those which were registered before the stimulation of the vagus. The depression gradually disappears.

Fig. 1b, which was obtained on Feb. 28, 1950 (lead II) shows a sinus rate of 115. This time only the right vagus nerve had been severed. Following faradic stimulation of the right vagus nerve for 2.4 seconds the first P wave again has an abnormal form but succeeding P waves, which look like those before the stimulation, are followed by a depression of the P-R segment which slowly diminishes and finally disappears.

These changes were observed in all of 17 experiments. They were barely recognizable in lead I, more marked in lead III and very distinct in lead II. Repetition of the stimulation of the vagus nerve again elicited this phenomenon without evidence of fatigue. We did not observe any difference in the effect of right or left vagus stimulation. The number of beats showing the depression of the P-R segment after the end of the stimulation varied with the rate.

We did not gain the impression that depression of the P-R segment was sustained longer by more prolonged stimulation of the vagus nerve. Thus in one experiment with a sinus rate of 176, vagus stimulation for 2 seconds caused a P-R depression persisting for 9 sinus beats; about 20 seconds later, after stimulation for 4.6 seconds, the same changes were seen for 12 beats; after stimulation for 8.4 seconds the P-R segment was depressed for 9 beats and about 25 seconds later, after stimulation for 9.6 seconds, changes were present for 8 beats; finally after a stimulation for only 3.8 seconds, the changes of the P-R segment were observed in 7 beats.

Intravenous injection of 0.005 g of acetylcholine chloride "Roche" invariably caused similar changes. After a temporary cardiac standstill, sinus rhythm reappeared and, as Fig. 2 shows (lead II), there was a marked depression of the P-R segment which quickly diminished and disappeared. This depression of the P-R segment can be attributed to the appearance of a pronounced Ta wave. It is known that a very distinct Ta wave may deform also the RS-T segment. We did not observe this, however, in our experiments, because the accentuated Ta wave following stimulation of the vagus nerves was of short duration. The steep return of the depressed P-R segment to the zero line demonstrates this fact. Shortening of the P-Ta interval during stimulation of the vagus nerve in frogs and mammals has been described(1,2).

Discussion. In the classic studies on the effect of stimulation of the vagus nerves on the electrocardiogram, these changes have

* This investigation was supported (in part) by a research grant from the National Heart Institute, U. S. Public Health Service.

1. Cohn, A. E., and McLeod, A. G., *Am. Heart J.*, 1939, v17, 305.

2. Cohn, A. E., and McLeod, A. G., *Am. Heart J.*, 1941, v21, 356.

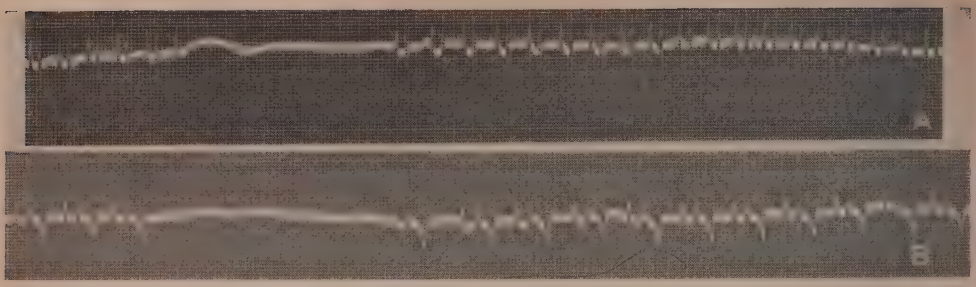

FIG. 1.
Fig. 1a shows a depression of the P-R segment during sinus tachycardia; Fig. 1b shows the same phenomenon during a normal sinus rate.

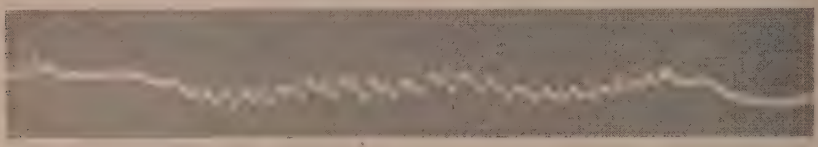

FIG. 2.
Shows the depression of the P-R segment following intravenous injection of acetylcholine.

escaped notice of the investigators. The phenomenon described above can easily be found in some of the tracings reproduced in their reports(3,4). Only in electrograms obtained with the exploring electrode within the right auricle of cats and dogs a similar deformity of the P-R segment has been registered. Standard leads are not described(2). In an investigation of the action of adenine nucleotides on the heart, Green and Stone stress the marked increase of vagus tonus after the intravenous injection of these compounds. Simultaneously with a marked inhibition of the A-V conduction, Fig. 29 and 30 of the monograph by these authors show a very distinct depression of the P-R segment in lead II(5). While our experiments were in progress, an accentuation of the Ta wave caused by acetylcholine was reported (6). These changes of the Ta wave are also found in clinical tracings. They appear in normal subjects after exercise(7). They are found for a few beats after carotid pressure. A similar depression of the RS-T segments has been described repeatedly in cases of coronary thrombosis and atrial infarction. The appearance of these changes by reason of a high vagal tonus has therefore some practical importance.

In an attempt of an explanation of this phenomenon certain experimental studies are pertinent. In an investigation of the effect of vagus stimulation on the conduction within the mammalian auricle an extremely rapid recovery was found during stimulation of the vagus nerve(8). The simultaneous reduction of the length of the refractory period is well known. Distinct shortening of the regression process was described(1,2), with the heart of the frog and in the mammalian heart after administration of acetyl-beta-methylcholine. Finally, Churney, Ashman and Biggins, in a study of the effect of the vagus on the monophasic action potential of the auricular

3. Hering, H. E., *Arch. f. d. ges. Physiol.*, 1909, v127, 155.

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5. Green, H. N., and Stoner, H. B., Biological actions of the adenine nucleotides, Lewis & Co., London, 1950.

6. Liebow, I. M., and Hellerstein, H. K., *Fed. Proc.*, 1950, v9, 77.

7. Scherf, D., and Goldhammer, S., *Z. f. klin. Med.*, 1933, v124, 111.

8. Drury, A. N., and Regnier, M., *Heart*, 1928, v14, 263.

muscle of the turtle, found a decrease in spike voltage due to a partial depolarization of the heart muscle fibers and an accelerated repolarization following vagal stimulation(9). A shortening of the repolarization process in the auricle may therefore be assumed to be responsible for the appearance of the Ta wave.

Conclusions. Vagal stimulation leads to the temporary appearance of a very pronounced Ta wave in the standard leads, particularly in lead II. This is explained by the abnormal repolarization process of the auricular muscle during and shortly after the stimulation.

9. Churney, L., Ashman, R., and Biggins, C. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 123.

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Anticomplementary Properties of Bone Marrow in Certain Diseases (17905)

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A low blood serum complement is often found in patients suffering of widespread disease of the bones(1). Because of this observation it was thought of interest to investigate the complement activity of the serous fluids expressed from the coagulate obtained from sternal bone marrow. Throughout this study the sternal puncture technic of v. d. Merwe(2) was employed. After the induction of local anesthesia with 2% novocain, the corpus sterni was punctured at the level of the second intercostal space. As a rule, the first drops obtained were smeared on slides for direct microscopic examination. After this, from 0.5 to 1 cc of the sternal marrow was withdrawn, ejected into a small serological test tube, and allowed to coagulate. After clotting the material was centrifugalized at 3000 r.p.m. for a period of 10 minutes and the supernate removed. For complement assay the hematocrit method of Jordan was used. One tenth of one cc of the fluid was employed and the titer obtained was compared with that of the blood serum which was tested at the same time.

It was found that in most cases the complement activity of the sternal fluid compared favorably with that of the blood serum. However, a small number of marrow fluids showed no complement activity at all while their corresponding sera possessed normal complement activity. It seems unlikely that these fluids should lack complement because a certain amount of blood is always obtained during puncture. It was found that the addition of complement components from normal serum failed to reactivate these marrow fluids. When these fluids were however mixed with active normal sera, they inhibited the complement activity of the latter. It was not feasible to work with larger quantities of sternal marrow since too great an admixture of blood would occur. It was observed that the anticomplementary power of these fluids resisted temperatures of 56°C and even 60°C so that it is possible to consider the anticomplementary activity as relatively thermostable (Olhagen)(3). In contrast to Olhagen's anticomplementary factor or factors the anticomplementary activity of these fluids is not acquired after heating of the fluids. In other words, they exist in the unheated fluid. In a number of patients whose sera and sternal marrows showed no complement activity, the sternal fluids were also tested for anticomplementary effects but none were observed.

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To date 126 patients have been studied. Of these, 15 showed the anticomplementary activity described above. In 19, complement activity was not entirely lacking but was found to be less than that observed in their blood sera. In 6, the complement level of the sternal fluid was definitely higher than that of the corresponding blood serum.

Of interest is the case of a man, 24 years of age, hospitalized because of a thrombosis of the splenic vein. The day before splenectomy was performed it was found that his sternal marrow fluid exhibited no complement activity while his blood serum gave a normal titer of 33. Immediately after the operation his serum complement level was 25. On the day following the operation his sternal marrow fluid complement rose from 0 to 32, and was of almost the same magnitude (35) as that of the blood serum drawn at that time. One month, postoperatively, the values were 58 and 51, respectively. (Fig. 1). A leucopenia and thrombocytopenia observed prior to operation disappeared after the splenectomy. Correlating this observation with the condition of the spleen in 126 patients it was discovered that they can be divided into two groups: (1) includes patients in whose sternal fluids complement levels are less than 20% of their corresponding blood sera values, and (2) comprises those cases in whom the sternal complement values do not deviate more than 20% from those obtained from their corresponding blood sera.

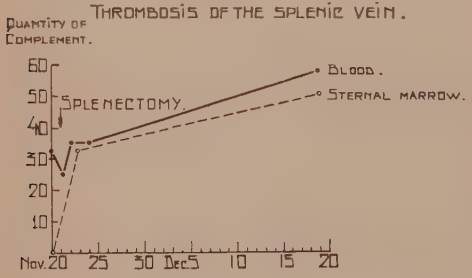

FIG. 1.

In Group I (34 patients), 21 showed an enlargement of the spleen. In Group II (86 patients), 17 exhibited an enlargement of the spleen. It can therefore be stated that in this series of cases, 60% of the patients showing anticomplementary activity of their sternal marrow fluids possessed an enlarged spleen, while in the second group splenic enlargement occurred in only 20% of the cases.

In 6 cases the complement values of the sternal marrow fluids were found to be 20% higher than those of their blood sera. In 4 of these 6 cases an enlargement of the spleen was observed.

Though the number of patients examined is relatively small statistically, it may be stated that Group I was composed of cases suffering of malignant granuloma, cirrhosis of the liver, Werlhof's disease, myeloid leukemia, thrombosis of the splenic vein, hemolytic icterus, and Besmer-Boeck-Schaumann disease. It is therefore likely that the peculiar behavior of the sternal marrow fluid is not a characteristic of a given disease, but shows some relationship to disorders of the spleen.

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Milk Agent and Natural Antisheep Agglutinins in Mice of Inbred Strains.* (17906)

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Higher incidence and higher titers of natural antisheep agglutinins have been found in C57 Black mice than in mice of other inbred strains. The same strain manifested also a better response to immunization with sheep

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TABLE I.
Antisheep Agglutinins and Milk Agent.

Strain		Total No.	Titer 0	1	2	4	8	16	32	Incidence of agglutinins, %
Z(C3H)	with milk agent	52	48	1	2	0	1	0	1	7.7
Zb	without milk agent	71	64	4	2	1	0	0	0	9.9
A	with milk agent	32	27	5	0	0	0	0	0	15.6
Ax	without milk agent	107	78	14	10	1	2	0	2	27.1
D_2	with milk agent	66	57	6	2	0	1	0	0	13.6
D_2	without milk agent	49	41	4	3	0	1	0	0	16.3
I	without milk agent	81	67	9	4	1	0	0	0	17.3

and human erythrocytes(1,2). Several factors have been examined as to their possible relationship to the difference in hemoantibodies(3). It was deemed advisable to investigate whether the presence or absence of the milk agent in the same strain is accompanied by a different incidence of natural antisheep agglutinins.

The animals were all bred in the Division of Cancer Biology, University of Minnesota Medical School, Minneapolis, with the exception of a small number of strain A mice, which were purchased from the Jackson Memorial Laboratory. All animals were at least 3 months old; the oldest were 16 months old. The technic of bleeding and of agglutination tests was the same as reported in previous work(1,2).

Table I lists 3 strains in which natural antisheep agglutinins were determined parallel in series of animals with and without the milk agent. Of these strains animals with the milk agent of strain C3H (Z) had been examined previously(1,2). The results obtained in mice of strain C3H (Z) showed significantly lower incidence of sheep agglutinins than the older material(2); possibly sublines of the same strain may differ in the occurrence of the antibodies(3). Neither in strain C3H (Z) nor in strains A and D_2 was there any significant difference in the incidence of sheep agglutinins between those groups which carried the milk agent and those free of the agent. The table shows also the results obtained on mice of strain I, which does not carry the milk agent. A low incidence of agglutinins was found in this strain.

Of the three new strains (A, D_2, I) examined in the course of this work, spontaneous mammary cancer occurs with considerable frequency in strains A and D_2. Mammary cancer incidence as well as incidence of other malignant tumors is low in strain I. Hence no relationship was demonstrated between the inherited susceptibility to mammary cancer and the frequency and titers of natural antisheep agglutinins.

Conclusion. The results of this study, as well as earlier findings(2) of a low incidence of antisheep agglutinins in animals of strains B alb C and Akm, which do not carry the agent, show that there is no relationship between presence or absence of the milk agent in mice and the incidence of natural antisheep agglutinins.

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Interrelationships of Desoxycorticosterone, Cortisone and Vitamin C in the Genesis of Mesenchymal Lesions.* (17907)

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Degenerative and atrophic changes in the collagen of the heart and joints resembling those of rheumatic fever and rheumatoid arthritis have been experimentally elicited by vitamin C deficiency(1,2,3) and by treatment with desoxycorticosterone acetate (DCA)(4). On the other hand, it has been shown that adrenocorticotrophin (ACTH) and cortisone can prevent or cure lesions of experimental(5) or clinical(6,7) collagenous diseases. Vitamin C is known to be directly implicated in the activity of the adrenal cortex(8,9,10,11) so that it seemed possible that some of the mesenchymal alterations in scurvy were not due to deficiency of vitamin C as such, but resulted from hyposecretion of cortisone-like steroids(12). The experiments here reported were designed to test this hypothesis on animals susceptible to vitamin C deficiency and to add to what is known of the contrasting functions of DC-like and cortisone-like steroids.

Experimental. Twenty-six male guinea pigs weighing between 280 and 300 g were divided into 4 groups and placed on a scorbutogenic diet as used by Schultz(1). There were 6 animals in each of the first 3 groups and 8 in the last one. The guinea pigs of Group I received a daily dose of 5 mg of DCA; those of Group II received a daily dose of 5 mg of cortisone acetate (Cortone acetate, Merck and Co.) which was increased to 7.5 mg on the 11th day and to 10 mg on the 19th day of the experiment; those of Group III were given *per os* 5 cc of orange juice daily and kept as normal controls; those of Group IV consisted of untreated scorbutic animals. The steroids in the form of aqueous suspension were administered subcutaneously, the daily dose being given in one injection. The animals were housed in cages of 3 and given 1% saline to drink as a sensitizing agent to the effects of DCA. Since it has been shown previously(1) that alterations in connective tissue are more manifest in chronic scurvy than in the acute disease, animals of Groups I, II and IV were given 1 cc of orange juice on the 4th, 8th and 11th day of the experiment. The course of the scurvy was evaluated from 4 criteria: general habitus, body growth, onset of arthritis and subcutaneous hemorrhages. The joint examined was the one formed by the ulna, radius and carpal bones. This area was epilated first with barium sulfide; then starting on the 10th day, measurements were taken at regular intervals with a caliper. On the 14th and 17th day X-ray pictures of the joints

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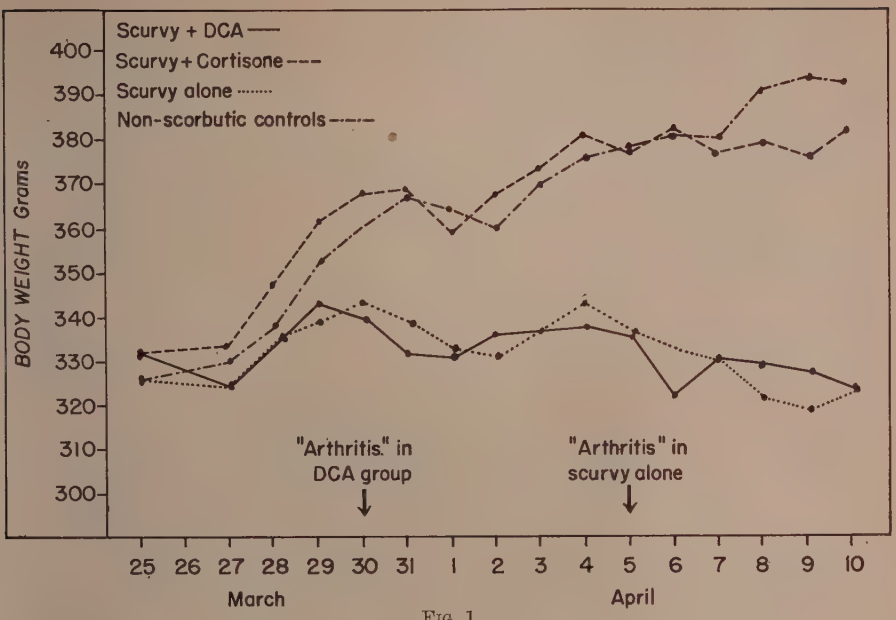

FIG. 1.
Effect of DCA and cortisone on body weight in the scorbutic guinea pig.

were taken on all the animals. The experiment was terminated on the 21st day.

The guinea pigs were anesthetized with nembutal and blood was drawn from the carotid artery for chemical studies. At autopsy adrenals, heart, kidney, pituitary, sternum and front paws were removed, fixed in 10% formalin or Zenker's fluid, weighed and sectioned. Histological data will form the subject of a subsequent report.

Results. 1. *General habitus.* Only animals of Group I (DCA) and Group IV (scorbutic controls) presented symptoms of scurvy; these appeared first and were more severe in the DCA group. On the 10th day, these animals began to show a decreased muscular activity, became apathetic and their fur dull and dirty; by the 15th day, most of them were prostrate and dyspneic. Two guinea pigs of Group IV died on the 15th and 17th day. The condition of the others was deteriorating rapidly, so that the experiment had to be terminated on the 21st day. The cortisone-treated guinea pigs did not differ in behavior from the non-scorbutic controls.

2. *Body Weight.* As can be seen from Fig. 1, animals of Groups I (DCA) and IV (scorbutic controls) started to lose weight at the same rate on the 10th day of the experiment, and continued to do so until the end. On the other hand, animals of Groups II (cortisone) and III (normal controls) gained weight in a parallel fashion, except at the end when the cortisone group barely maintained its gain.

3. *Joint lesions.* The effects of DCA and cortisone on the development of articular

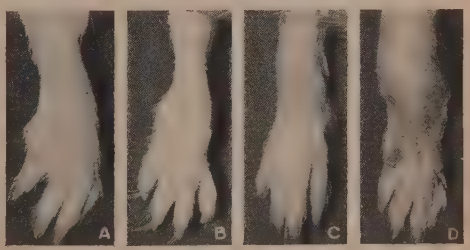

FIG. 2.
Right front paws taken on the 16th day of the experiment. A—Normal control. B—Cortisone-treated scorbutic guinea pig. C—Untreated scorbutic guinea pig. D—DCA-treated scorbutic guinea pig.

TABLE I.
Morphologic Changes Produced by DCA and Cortisone in Scorbutic Guinea Pigs.

Treatment	Micrometer reading right front paw, mm Day 10th	Day 13th	Day 15th	Wt Adrenals, mg ± S.E.D.	Wt Heart, g ± S.E.D.	Wt Kidney, g (range)
I. Scurvy + DCA	7.58 ±.38	8.11 ±.25	8.51 ±.22	210 ±16.9	1.026 ±.043	1.666 (1.40-1.94)
II. Scurvy + cortisone	6.68 ±.23	6.56 ±.10	6.44 ±.02	136 ± 9.7	1.447 ±.015	1.922 (1.64-2.14)
III. Normal controls	6.76 ±.07	6.82 ±.06	6.91 ±.08	158 ±16.9	1.310 ±.120	1.830 (1.52-2.05)
IV. Scorbutic controls	6.71 ±.21	7.11 ±.28	7.30 ±.28	236 ±26.5	1.032 ±.165	1.713 (1.32-2.00)
P values*		I-III .01	I-III .01	III-IV .05		
		I-IV .02	I-IV .01	I-IV .4		
				II-IV .01	II-IV .05	

* P values of less than 0.05 are considered statistically significant.

lesions due to scurvy were very striking (Fig. 2). In Table I, we list measurements of the radial-carpal joint taken at various intervals. Guinea pigs in Groups I and IV developed typical swelling accompanied with redness and elevation of temperature; it became progressively more severe in the course of the experiment. While there was no obvious qualitative difference in the joint swelling of the DCA and the scorbutic animals, it must be pointed out that it appeared sooner in the DCA-treated (10th day) than in the scorbutic control (15th day) guinea pigs, and, that on the 15th day it was present in 5 out of 6 animals in the former group and only in 3 out of 8 in the latter. Furthermore, measurement values of the joints, summarized in Table I, show that the difference between the 2 groups became significant on the 13th day. Cortisone prevented these lesions throughout the experiment in all except one guinea pig. This animal developed pronounced and diffuse swelling in all four limbs, extending beyond the limits of the joints. However, it continued to gain weight and at autopsy the organs were found to be normal. Since these articular and periarticular lesions seemed to be of a different nature than those seen in the scorbutic controls, they were omitted from Table I.

4. *Hemorrhages.* With the exception of the normal controls, all other groups showed diffuse hemorrhages at autopsy. These were mainly in the subcutaneous tissue, the muscles and the joints. They were less severe in the cortisone group and more so in the DCA group. One guinea pig treated with cortisone did not show hemorrhages.

5. *Organ weights.* Weights of adrenals, heart and kidney are summarized in Table I. Scurvy caused a marked adrenal hypertrophy which was completely prevented by cortisone but only partially suppressed by DCA. The adrenals of the DCA-treated guinea pigs were somewhat smaller than those of the scorbutic controls and showed subcapsular and parenchymal hemorrhages. The weight of the heart tended to be less in the DCA and the scorbutic animals than in the cortisone and the normal control groups. These changes, however, were not significant. There were no obvious differences in kidney weight between the various groups. It should be noted, however, that the kidneys of the

DCA group presented patchy areas of pallor.

Determinations of arterial pressure, serum CO_2 content, chloride and alkaline phosphatase in some animals of each group did not indicate any striking difference. Total blood glutathione was kindly measured in all by Dr. A. Lazarow according to a method based on the formation of a complex glutathion-alloxan(13). It was decreased in all the experimental groups; this was most marked in the cortisone-treated animals. The data correspond with observations on normal rats similarly treated(14).

Discussion. It has been postulated(12) that most of the symptoms of experimental scurvy are secondary to adrenal insufficiency; this is based on certain similarities between the two conditions: (1) clinical symptoms (asthenia, adynamia, loss of weight) (2) disturbance in water (edema), salt (potassium retention), carbohydrate (decrease in liver glycogen and phosphocreatin) and protein metabolism (increase in blood urea nitrogen). Later it was found that the adrenal corticosteroid content is markedly diminished in vitamin C deficiency(15,16,17) and that symptoms of scurvy can be partially corrected with cortical extract(8,9) but not with desoxycorticosterone(18). The present experiments establish with crystalline cortisone the anti-scorbutic properties of carbohydrate active corticosteroids. The aggravation of the symptoms of scurvy under the influence of DCA emphasizes an antagonism between cortisone and DCA which has been demonstrated in other respects by previous investigators. Adreno-cortical hypertrophy during vitamin C depletion has been previously observed(19,20,21). This hypertrophy is presumably due to a compensatory ACTH overproduction resulting from a decrease in circulating adreno-cortical steroids. That cortisone was able completely to prevent this hypertrophy while DCA showed little effect, favors the view that the factors responsible for the secretion of these two types of cortical hormones are independent.

These experiments are of interest in the light of recent findings on the effect of cortisone and ACTH in collagenous diseases(6,7). The fact that most of the important evidences of scurvy were prevented by cortisone administration may elucidate the mode of action of this hormone in rheumatic conditions. Thus, in viewing scorbutic arthritis, vitamin C deficiency may be supposed to lead to insufficiency of carbohydrate-active corticosteroids; the lack of these may underlie the collagen degeneration which in turn results in arthritis. The deleterious effects of exogenous desoxycorticosterone is consistent with the view that the arthritis of untreated scorbutic animals may be due to cortical carbohydrate-active deficiency with preservation of production of desoxycorticosterone-like compounds, that is to a hormonal imbalance. The fact that the zona glomerulosa, where desoxycorticosterone is produced (22) is very poor in its content of vitamin C(23) while the fasiculata and reticularis are richer, would indicate that ascorbic acid may not be essential in the same degree for the production of these two types of cortical steroids.

Cortisone was unable to prevent some of the evidences of scurvy, notably the capillary hemorrhages. Very likely also it would not have indefinitely stayed the weight loss, at least at the dose employed in these experiments. Conceivably there may be in the

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adrenal cortex other steroids which may be more effectively anti-scorbutic than cortisone. More probably, some of the activities of vitamin C may be independent of the adrenal cortex.

The similarities between scurvy and rheumatic conditions are in many respects very striking. Consequently, the DCA-treated scorbutic guinea pig or the chronically scorbutic animal would seem to be excellent test subjects for the screening of compounds with anti-rheumatic activity. The advantage of such a test animal is the more evident as it now becomes apparent that adrenal glycogenic and anti-arthritic properties can be dissociated.

Summary. Cortisone inhibits many of the manifestations of scurvy in the guinea pig while desoxycorticosterone aggravates the condition. These activities of the hormones probably depend on their action on mesenchymal tissues. The scorbutic guinea pig, untreated or treated with DCA, may be a valuable test subject for the assay of anti-arthritic compounds.

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Effect of Tolerance on Inhibition of Respiration of Brain Homogenates by Thiopental. (17908)

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(Introduced by Paul K. Smith)

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In a previous paper it was shown that mice become tolerant to the daily intraperitoneal injection of thiopental sodium, 45 mg per kg. This tolerance became maximal in 5 to 6 days and was indicated by shortened sleeping times and the ability of the experimental animals to awaken at higher brain levels of the barbiturate than controls(1). Since it has been proposed that barbiturates induce sedation by interference with the carbohydrate metabolism of the brain(2,3,4) it seemed important to study the inhibitory effects of barbiturates on the *in vitro* carbohydrate metabolism on the brains of control and tolerant mice.

Experimental. Male mice, weighing 23 to 25 g were made tolerant by the daily intraperitoneal injection of thiopental sodium, 45 mg per kg, for from 7 to 10 days; controls, kept under identical conditions, were given daily injections of saline. Records of the sleeping times of the experimental animals were kept, and the animals were used when their sleeping times were between 50 and 60% of their original sleeping times. For each experiment the whole brains from 5 control and 5 tolerant mice were rapidly removed and homogenized in cold distilled water in a glass homogenizer. No more than 15 minutes were allowed to elapse from the time the animals were killed until the homogenates were placed in the reaction vessels. The method of Reiner (5) was used in the study of glucose, pyruvate, and lactate oxidation. Since it seemed possible that the addition of the cofactors in these fortified homogenates might mask some difference in the metabolism of the control and tolerant mouse brains, isotonic homoge-

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TABLE I.
Effect of Thiopental on the Respiration of Brain Homogenates from Control and Tolerant Mice.

Thiopental sodium conc. (mg per cc)		0	83.3	166.6	333.3	666.6
Glucose oxidation	Control	6.22*	6.15	4.63	3.11	1.86
	Tolerant	6.14	6.21	4.69	3.06	1.73
Pyruvate oxidation	Control	7.80	7.68	4.68	3.12	1.90
	Tolerant	7.67	7.77	4.53	3.16	1.79
Lactate oxidation	Control	6.88	6.80	4.51	3.03	1.76
	Tolerant	7.02	6.88	4.38	2.97	1.88
Glucose anaerobic glycolysis	Control	5.55†	5.60	5.49	5.53	5.34
	Tolerant	5.39	5.55	5.62	5.60	5.42
Succinate oxidation	Control	3.85	3.78	3.80	3.70	3.89
	Tolerant	3.74	3.86	3.69	3.89	3.66
Cytochrome oxidase	Control	3.35	3.43	3.46	3.38	3.40
	Tolerant	3.39	3.30	3.33	3.30	3.36

* Values are given as ml O_2 per mg of wet wt of tissue per hr.

† Values for anaerobic glycolysis are in ml CO_2 per mg of wet wt of tissue per hr.

Temp. 36.3°C; gas phase, air; 0.1 cc of 30% KOH in center well; conc. of thiopental sodium as shown above. Contents of flasks; (a) Glucose oxidation—glucose .028 M; hexose diphosphate Ba, .005 M; ATP, .0007 M; DPN, .001 M; nicotinamide, .04 M; cytochrome C, .00006 M; K_2HPO_4-KH_2PO_4 buffer, pH 7.4, .04 M; .5 cc 1:4 mouse brain homogenate; final volume 3 cc. (b) Pyruvate and lactate oxidation—pyruvate .028 M, or lactate .028 M in place of glucose, otherwise as in (a). (c) Anaerobic glycolysis of glucose—Temp. 36.3°C, gas phase 95% N + 5% CO_2; conc. of sol.: glucose, .028 M; hexose diphosphate, Ba, .0025 M; ATP, .0007 M; DPN, .0005 M; nicotinamide, .04 M; K_2HPO_4-KH_2PO_4 buffer, pH 7.4; .01 M; $NaHCO_3$, .048 M; 5 cc 1:4 mouse brain homogenate. (d) Succinate oxidation—sodium succinate, .05 M; cytochrome C, .00017 M; $CaCl_2$, .0004 M; $AlCl_3$, .0004 M; Na_2HPO_4-HCl buffer, pH 7.4; .03 M; 1 cc 1:4 mouse brain homogenate; final vol. 3 cc. (e) Cytochrome oxidase-sodium ascorbate, .0114 M, in place of succinate, otherwise the same as (d).

nate preparations were also used to study glucose oxidation. These were prepared by homogenizing the brain in Ringer's phosphate buffer solution and using 0.028 M glucose as substrate. The method of Utter, Wood, and Reiner(6) was used for the study of anaerobic glycolysis; and that of Schneider and Potter(7) for the study of succinate dehydrogenase and cytochrome oxidase systems. The rate of oxygen uptake or carbon dioxide evolution was followed in a Warburg manometer at 36.3°C. After a 15-minute period for gassing and temperature equilibration the stopcocks were closed and readings were taken at 10-minute intervals for one hour. All determinations were made in duplicate, and each experiment was repeated several times on different groups of control and experimental animals. The values in Table I represent the average of 3 or more separate experiments.

In a second study, 10 control and 10 tolerant mice were given thiopental sodium, 85 mg per kg intraperitoneally (approximately an $L.D._{50}$ dose), the mice in each group sacrificed after 5 minutes, and the brains prepared for *in vitro* respiration studies as above.

Results. Table I summarizes the results of the experiments. Thiopental in concentrations of from 167 mg per cc to 667 mg per cc produced from 30 to 80% inhibition of the oxidation of glucose, pyruvate, and lactate. However, the control and tolerant mouse brains showed no difference in the rate of oxidation of these substrates, nor in the degree of inhibition caused by thiopental on the oxidation of these substrates. There was no difference in the anaerobic glycolysis of glucose, the succinic dehydrogenase or the cytochrome oxidase activity. None of

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these systems in either control or tolerant brain preparations were inhibited by the thiopental concentration used. The results obtained with the oxidation of glucose using the isotonic homogenate preparations were essentially the same as those shown in Table I. In studies in which an $L.D._{50}$ dose of thiopental was injected into the animals, rather than added to the flasks, no inhibition of glucose oxidation was observed in control or tolerant brain preparations.

Discussion. If the sole mechanism of action of barbiturates in producing hypnosis is a depression of the carbohydrate metabolism of the brain, then it should follow that tolerant animals, requiring a higher brain concentration of the barbiturate to maintain hypnosis, should show less inhibitory effects of the barbiturate on brain respiration than normals. This was not found to be true. If such differences had been present, it should have been possible to demonstrate them by the technics used, since the brains of control animals are almost twice as sensitive to the hypnotic effects of thiopental as those of tolerant animals. Further, the minimum *in vitro* concentration of thiopental required to inhibit glucose oxidation by brain homogenates is equivalent to an *in vivo* brain concentration which we have found to be uniformly lethal(8). Our observation that the injection of an $L.D._{50}$ dose of the barbiturate in mice failed to affect the *in vitro* carbohydrate metabolism of the brain substantiates similar observations of others(2). However, since slices were used by these workers, failure to observe inhibition was attributed to diffusion and dilution of the drug in the surrounding medium. The use of the homogenate technic circumvents this difficulty since the enzyme and drug are equally diluted. Several reports in the literature comparing hypnotic doses of barbiturates *in vivo* and drug concentrations required for inhibition of brain respiration *in vitro*(9,10) are based on the contention (as reported by Tatum *et al.* for amytal in rabbits)(11) that the brain contains four times as much of the barbiturate as other tissues. Accurate spectrophotometric methods for the determination of barbiturates in tissue have shown that most of the barbiturates do not reach higher concentrations in the brain than in most of the other parenchymatous tissues(12). It appears unlikely that the concentrations of barbiturate equivalent to those found in the brain during hypnosis would inhibit *in vitro* glucose metabolism by brain. It should also be noted that pyribenzamine(13) and some of the local anesthetics(2,14) similarly depress the *in vitro* metabolism of glucose, but are convulsant when administered in equivalent concentrations *in vivo*. These observations do not rule out the possibility of selective activity of barbiturates on some local area of the brain so sensitive that the effects could not be demonstrated on whole brain preparations, though *in vitro* studies have shown no difference in the sensitivity of various areas of the brain to pentobarbital(15).

Summary. 1. No difference was demonstrable in the rate of *in vitro* oxidation of glucose, lactate, pyruvate and in the succinic dehydrogenase and cytochrome oxidase systems by brain homogenates from control and thiopental-tolerant mice.

2. Thiopental was found to inhibit the *in vitro* oxidation of glucose, lactate, and pyruvate but there was no difference in the degree of inhibition between control and tolerant mouse brain homogenates. The anaerobic glycolysis of glucose and the activity of succinic dehydrogenase and cytochrome oxidase were unaffected by the thiopental.

3. No inhibition of the *in vitro* oxidation of glucose was observed in the brain homog-

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14. Watts, D. T., *J. Pharm. and Exp. Therap.*, 1949, v96, 325.

15. Gerard, R. W., *Ann. Rev. Biochem.*, 1937, v6, 419.

enates from normal or tolerant mice after receiving an $L.D._{50}$ dose of thiopental.

4. It is concluded that the observed *in vitro* depression of brain respiration by barbiturates cannot be the sole mechanism responsible for barbiturate hypnosis.

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A Method for Quantitative Estimation of Small Amounts of D-Tubocurarine Chloride in Plasma.* (17909)

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To date no chemical method for the determination of D-tubocurarine in plasma has been reported. A method by which two micrograms of D-tubocurarine chloride per ml of plasma can be estimated has been developed and is presented along with data on the plasma levels associated with paralysis in the rabbit. The principle of the procedure is that employed by Brodie and Udenfriend (1) for many organic bases, that is, extraction of the alkaloid into an organic solvent and subsequent complex formation with methyl orange. D-tubocurarine cannot be extracted from alkaline solution into organic solvents. It may, however, be extracted from a potassium iodide solution at pH 10. Other salts were unsatisfactory because they caused the formation of emulsions between the plasma and solvent, gave high blanks, or failed to result in complete recovery.

Reagents. Ethylene dichloride. Because this solvent usually contains impurities which react with methyl orange, it was purified in one of 2 ways: (a) by treating with activated charcoal, Norite, or (b) by washing once with one-fifth volume of N hydrochloric acid and then several times with water. The purified solvent was stored in a glass-stoppered bottle. *Methyl Orange.* One gram of the sodium salt of methyl orange was dissolved in 500 ml of water and the resulting solution was shaken several times with equal volumes of purified ethylene dichloride to remove soluble organic impurities. The free acid of methyl orange was then precipitated by the addition of 2 ml of concentrated sulfuric acid, filtered on a Buchner funnel, washed several times with water and dried *in vacuo*. *Acid-alcohol.* Two ml of concentrated sulfuric acid were dissolved in 100 ml of absolute ethanol. *Potassium-iodide-glycine buffer.* A buffer solution of about pH 10 was prepared by mixing 6.0 ml of 0.1 M glycine solution containing sodium chloride (7.505 g glycine and 5.85 g sodium chloride per liter) with 4 ml of 0.1 N sodium hydroxide. To 10 ml of this buffer, 12.8 g of reagent grade potassium iodide were added and the resulting solution was kept in a brown bottle to protect it from the light. The buffer is very stable but the buffer-potassium-iodide solution is not and the mixture should be prepared daily. *Citric acid buffer.* A citric acid buffer solution, pH 5.0 was prepared by mixing 10.3 ml of 0.2 M dibasic sodium phosphate with 9.7 ml of 0.1 M citric acid.

Procedure. Three ml of plasma containing 5 to 60 micrograms of D-tubocurarine chloride are pipetted into a 60 ml glass-stoppered bottle containing 10 ml of ethylene dichloride. One-half ml of the potassium iodide-glycine buffer solution is added and the mixture is shaken for 5 minutes, decanted into a test tube, centrifuged and the aqueous

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1. Brodie, B. B., and Udenfriend, S., *J. Biol. Chem.*, 1945, v158, 705.

phase removed by aspiration. The ethylene dichloride phase is transferred to a glass-stoppered test tube, three drops of the citric acid buffer and a few crystals of methyl orange are added. The test tube with contents is shaken for 2 minutes, centrifuged and the excess dye solution is completely removed by aspiration with a finely drawn glass tube. Five ml of the ethylene dichloride containing the dye complex is transferred to a colorimeter tube and 0.5 ml of the sulfuric acid-ethanol solution is added. The optical density of the pink-colored solution is measured in a Klett-Summerson photoelectric colorimeter using a No. 540 filter with the zero adjusted using ethylene dichloride. In plasma and urine there are ethylene dichloride soluble substances which react with methyl orange. The blank obtained with one ml plasma is equivalent to about 0.06 μg of D-tubocurarine; that obtained with urine was so much higher that further investigation of urine samples was not attempted. Recoveries of 5 to 60 μg D-tubocurarine chloride added to plasma were satisfactory (Table I).

Standard curve. A stock solution containing 100 mg of crystalline D-tubocurarine chloride in 100 ml of water was kept in the refrigerator and dilute solutions prepared as needed. A standard curve was prepared by extracting various amounts of D-tubocurarine chloride, 5 to 60 μg, from water in the same manner as for the plasma determination. A reagent blank was tested by the same procedure. A linear relationship between the concentration of alkaloid and optical density was obtained. A net density reading of 60 was obtained on the Klett-Summerson photoelectric colorimeter for a concentration of 1 μg of D-tubocurarine chloride per ml of ethylene dichloride.

TABLE I.
Recovery of D-Tubocurarine Chloride from Plasma.

D-tubocurarine chloride added, μg	D-tubocurarine chloride found, μg	Recovery %
5	5.0	100
	5.0	100
10	10.0	100
	10.3	103
20	20.4	102
	20.0	100
30	30.3	101
	30.3	101
40	38.4	96
	40.0	100
50	50.0	100
	48.5	97
60	58.3	97
	58.3	97

Plasma level of paralytic dose of tubocurarine in rabbits and its pharmacological effect. Plasma levels of D-tubocurarine were correlated with the dose required to produce "head drop" in rabbits. The criterion for the "head drop" dose was the relaxation of the neck muscles so that the vertebrae could easily be felt; at this time the animal was unable to lift its head or move its legs and the respiration was slowed. On recovery, first the respiration returned to normal, followed by a resumption of tone in the neck muscles and then in the extremities. The animal appeared normal about ten minutes after the injection was stopped.

Both crystalline D-tubocurarine chloride and a partially purified preparation, Intocostrin, were used. Each preparation was made up in an isotonic solution so that each ml had a "potency equivalent to 20 units of standard curare by Rabbit-Crossover Test". For all experiments each solution was diluted ten times with saline so that each ml contained two units (0.30 mg per ml) and the rate of injection was maintained at 0.2 ml every 45 seconds. A control blood sample was taken by cardiac puncture before starting the infusion. Data from these experiments are shown in Table II.

Approximately equal amounts of D-tubocurarine chloride and Intocostrin were required to produce paralysis. It is interesting to note with each of these preparations that the plasma level was critical and constant, although no constant dose was required to produce "head drop". Thus, rabbit No. 3 was paralyzed with 0.221 mg per kg, and rabbit No. 4 required nearly twice as much, *viz.*, 0.408 mg per kg; nevertheless the plasma level in each case was 1.6 mg per liter.

Summary. Small amounts of D-tubocurarine can be measured in plasma by extraction with ethylene dichloride and reaction with

TABLE II.
Plasma Concentrations of D-Tubocurarine Found at Time of Rabbit Head-drop After Administration of D-Tubocurarine Chloride or Intocostrin.

Animal No.	D-Tubocurarine chloride		Intocostrin	
	Amt. inj. mg/kg	Plasma level at time of head drop mg/liter	Amt. inj. mg/kg	Plasma level at time of head drop mg/liter
5	.385	2.1	—	—
10	.264	2.2	—	—
6	.252	1.8	—	—
4	—	—	.408	1.6
3	—	—	.221	1.6
5	—	—	.333	1.5

methyl orange. The plasma level for paralysis in rabbits is about 1.5 to 2.2 mg per liter.

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Experimental Production of Sterile Closed Intestinal Loops. (17910)

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The purpose of this paper is (1) to report a method for the production of sterile closed intestinal loops, and (2) to report some subsequent observations upon these loops.

Methods. Several combinations of phthalylsulfathiazole and dihydrostreptomycin were used. However, the simplest and most reliable method consisted of the following. Healthy adult dogs were given, prior to surgery, 6.0 g of phthalylsulfathiazole each 12 hours for 3 days and 0.5 g of dihydrostreptomycin each 12 hours for 2 days before surgery. The animal was fasted for 24 hours and anaesthesized with pentobarbital and ether. All operative procedures were performed with strict aseptic technic. The ligatures and sutures were 000 silk. The abdomen was entered through a midline incision beginning at the xyphoid process and extending inferiorly for 4 inches. Both jejunal and ileal closed loops were made. The segment of either jejunum or ileum was chosen with a good blood supply that would not be embarrassed when brought through the abdominal wall and transplanted subcutaneously. Therefore the length of the loop and its distance from either the ligament of Treitz or the ileocecal valve varied in different dogs; the details are listed in Table I. Having determined the level of the loop and its length, the gut was transected in two places between hemostats. The loop was brought up through the abdominal incision, the continuity of the bowel reestablished by the aseptic anastomosis technic, and the linea alba was closed in a manner which would not interfere with the mesenteric pedicle supplying blood to the loop. The clamps were removed from the ends of the loop, and the loop irrigated five times with 200 cc of 1:1000 aqueous zephiran chloride followed by similar irrigations with sterile water. The ends of the loop were next closed by inverting with a purse string suture followed by interrupted inverting sutures. The closed loop was transplanted subcutaneously by undermining the adjacent skin edges. The skin was then closed by a running suture. Seven

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TABLE I.
Level of the Closed Loop, Its Length, and Time of Culture.

Dog No.	Distance from ligament of Treitz, inches	Length of loop, inches	Culture days post operative
		A. Jejunal closed loops	
81	13	9	7th day negative
84	25	14	10th " "
87	21	12	2nd day positive-*B. coli* 80 colonies per cc
90	26	7	13th day negative
			27th " "
91	20	9	13th " "
135	15	13	21st " "
136	15	10	21st " "
		B. Ileal closed loops	
Dog No.	Distance from ileo-cecal valve, inches	Length of loop, inches	Culture days post operative
94	16	9	8th day negative
			14th " "
			53rd " "
157	18	11	19th " "
			24th " "
114	19	20	8th day positive for *Clostridium welchi* and *B. proteus*

dogs with closed jejunal and 3 dogs with closed ileal loops were prepared. The loops were aspirated almost every day with a sterile needle and syringe. The secretions of the closed loops were cultured for both aerobic and anaerobic organisms by Dr. Ernest Witebsky of the Department of Bacteriology. The anaerobic cultures were observed for 5 days before giving a negative report.

Eight additional dogs with either a jejunal or ileal loop were prepared in a similar manner except that the ends of the loop were not closed but were introduced from their subcutaneous pocket into the peritoneal cavity through a rectus stab wound. The serosa around the periphery of the opening into the lumen was sutured to the peritoneum. A Crosby-Cooney button was inserted into the lumen of the loop and anchored with sutures to the peritoneum. This kept the peritoneum from closing over the lumen of the loop. Such a loop had free drainage into the peritoneal cavity. The animals were sacrificed from 2 weeks to 6 months later. The loop was examined histologically by fixing the tissue in 10% formaldehyde and staining with hematoxylin-eosin.

Results and observations. There were two failures to produce a sterile closed loop in the 10 dogs. The level of the loop, its length, and time of culture are listed in Table I.

The secretions aspirated from the closed loops were at first blood tinged but between the fourth and seventh day, clearing had usually taken place. The quantity of fluid aspirated daily usually varied from 50 to 75 cc for the first week. By the time the animal was taking a normal diet, the secretions increased to from 150 to 200 cc per day. However, with time secretions became less. This was particularly striking in dog No. 94 who in approximately 6 weeks was secreting only a few cubic centimeters of almost pure mucus. Two months after the closed loop had been made, 100 cc of 5% glucose in 0.9% NaCl was injected into the lumen. On the following morning fluid could not be reaspirated. A similar amount of 5% amigen was also absorbed. If 1% methylene blue was injected into the lumen it would appear in the urine. Peristalsis was always easily visible beneath the skin.

If the loop was not aspirated and allowed to distend, loss of appetite usually occurred. Appetite was rapidly regained if either the loop was aspirated or it ruptured.

The total protein content of the secretions of loop varied from 0.2 to 1.7 g %, and the

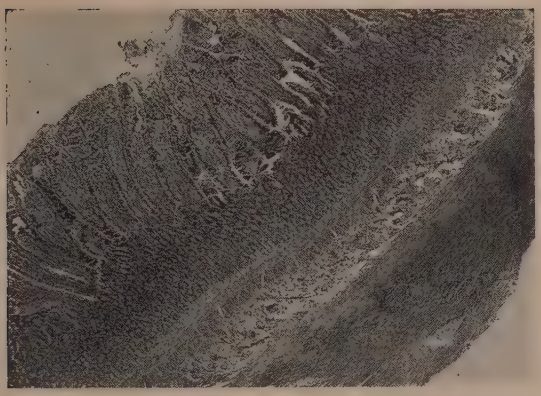

FIG. 1.
Dog No. 90. Cross section of intestine 88 days after closure. There is a normal mucosa and lack of inflammatory changes. 30 ×.

chloride content varied from 492 to 561 mg %.

In the 8 dogs in which the loop had free drainage into the peritoneal cavity there were no deaths, and no evidence of peritonitis when sacrificed up to 6 months after operation.

Discussion. As far as we know primary closed sterile loops have not been produced before, Dragstedt(1) however did sterilize loops by prolonged drainage into the abdominal cavity; such loops could be secondarily converted into closed sterile loops without untoward symptoms. Dragstedt(2) was unable to produce sterile loops by mechanical washing with the usual antiseptics and caustics. This was confirmed by studies of Wangensteen and Waldron(3). The fact that no deaths occurred in the series of animals in which one end drained into the peritoneal cavity we believe is significant. In Dragstedt's series of animals there was a 50% mortality when the gut was mechanically washed with the usual antiseptics followed by ether. The drainage of intestinal secretions into the peritoneal cavity for as long as 6 months had no untoward effect on the animal. It is not surprising that as time went on absorption from the loop exceeded secretions. It is well known that Thirry-Vella loops and gastric pouches secrete less after prolonged drainage. Clinically, we know that in iliostomies and colostomies, absorption gradually exceeds secretions. The reason for this is not well explained in the literature. Even though secretion became minimal in these loops it was significant to find absorption preserved several months after the loops had been made.

The animal with a closed sterile loop, at no time, showed signs of "toxemia" as has been described many times. Even when the intra-luminal pressure was allowed to increase sufficiently to cause rupture the "toxemia" syndrome was not observed.

Histological examination of each loop did not reveal abnormal findings. The mucosa and villi were intact and well preserved. There was no evidence of inflammation within the wall of the loop.

We believe from the evidence presented that closed sterile intestinal loops lend themselves to various physiologic or pharmacologic investigation. The length of time a loop will remain sterile is dependent upon aseptic aspiration.

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2. Dragstedt, L. R., Dragstedt, C. A., McClintock, J. T., and Chase, C. S., *J. Exp. Med.*, 1919, v30, 109.

3. Wangensteen, O. H., and Waldron, G. W., *Arch. Surg.*, 1928, v17, 430.

Summary. 1. A method is presented for the preparation of sterile closed intestinal loops. 2. The animal does not show signs of toxemia. 3. Evidence is offered that absorption continues in the sterile loop.

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Utilization of the Labile Factor During Normal and Abnormal Coagulation of Blood.* (17911)

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The study of the extent of utilization of the different factors known to be necessary for the formation of thrombin is a fruitful technic of investigation of the clotting process. Thus, in the hands of Quick and associates, the quantitative determination of the consumption of prothrombin has given conclusive evidence of the existence of a preliminary phase in the coagulation of blood, namely the formation of thromboplastin from an inactive plasmatic precursor through the action of a platelet factor(1). It has also helped to identify the mechanism of the coagulation defect in hemophilia and thrombocytopenic purpura(1,2). The factors taking part in the formation of thrombin are not totally utilized during coagulation. Examination of the serum by one-stage technics, after the thrombin formed has been neutralized by the natural antithrombin, reveals the presence of at least 3 proteins active in promoting the coagulation of blood: (a) unconverted prothrombin; (b) unconverted labile factor (factor V, plasma Ac globulin); and (c) a new agent, capable of accelerating the conversion of prothrombin to thrombin (serum Ac globulin, factor VI, spca, etc.). The concentration of these 3 factors varies according to the normality or abnormality of the coagulation process.

The present paper introduces data on the utilization of the labile factor during coagulation in healthy subjects and in patients with different disorders of the clotting mechanism. The relationship of the utilization of the labile factor to the consumption of prothrombin and to the development of the accelerator in serum has also been investigated, since previous findings have suggested that the accelerator may derive from the labile factor through the action of thrombin(3).

Materials and methods. A 20-gauge needle on a glass syringe was neatly inserted into a vein and a few ml of blood were drawn. The glass syringe was then exchanged for a second one coated with Silicone and chilled in ice prior to use. Four ml of blood, apparently uncontaminated with tissue thromboplastin, were collected and divided into 2 samples of 2 ml each. The first was decalcified by the addition of 1/10 volume of sodium oxalate 0.1 M and the second allowed to clot in a glass test tube in a water bath at 37°C. Plasma was obtained from the first sample by centrifugation at 1,500 r.p.m. for 5 minutes and its labile factor activity determined by the method of Quick and Stefanini(4) as described by Stefanini(5). This consists essentially in determining the ability of the plasma under observation to reduce towards normal values the

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TABLE I.
Consumption of Prothrombin During Coagulation and Its Relation to Utilization of Labile Factor and Development of Accelerator in Serum.*

Diagnosis	No. cases	Serum/plasma prothrombin activity ratio, ×100	Labile factor activity of plasma, %	Labile factor activity of serum, %	Serum/plasma labile factor activity ratio, ×100	Accelerator effect of serum, %
Normal	15	2.4	98	14.2	14.3	89
Polycythemia vera	10	4.3	100	19.6	19.6	87
Pseudohemophilia	4	5.1	100	11.3	11.3	91
Hemophilia	5	84.3	94.8	68.5	72.5	12
Thrombocytopenic purpura	7	75.0	97.0	74.2	75.9	15
Symptomatic thrombocytopenia	21	81.4	74.0	63.0	84.6	9
Dicumarol hypoprothrombinemia	10	11.4	92.6	45.4	49.2	24
Cirrhosis of liver	5	42.8	51.4	29.7	58.0	39

* Prothrombin activity, labile factor activity and accelerator were determined in serum one hour after the completion of coagulation.

prolonged prothrombin time of stored human oxalated plasma(4). The clotted sample was centrifuged at 1,500 r.p.m. for 2 minutes, one hour after the completion of coagulation and the serum was separated and treated with the addition of 1/10 volume of sodium oxalate solution 0.1 M and then absorbed with $Ca_3(PO_4)_2$ gel 0.008 M. This treatment was found necessary to neutralize the thrombin still present in the serum and to adsorb all prothrombin and accelerator agent from the serum. The removal of the latter substance was found to be erratic and incomplete if the serum was not oxalated prior to adsorption. The labile factor of the supernatant serum was then determined with the previously described method(5). The concentration of serum accelerator was determined with the one-stage method of deVries *et al.*(6); the plasma prothrombin activity with the method of Quick(7); the prothrombin activity of the serum with the method recently described, in which correction is made for the accelerator effect of serum(8). In this paper we give the ratio between prothrombin activity of serum and prothrombin activity of plasma, which is a more correct indication of the consumption of prothrombin during coagulation than the original values. The ratio was calculated by dividing the values of the serum and plasma prothrombin activities and multiplying the result × 100 (8). Healthy subjects and patients with different disorders of the hemostatic mechanism were investigated. The number of individuals studied for each condition is indicated in Table I. Cases of polycythemia vera and pseudohemophilia were included in this series to show the relation of the utilization of the labile factor to the utilization of prothrombin and development of accelerator in serum in conditions in which the bleeding tendency is not due to demonstrable deficiency of the coagulation mechanism.

Results and discussion. The findings are summarized in Table I. About 85% of the labile factor was utilized during coagulation in healthy subjects and smaller amounts in those presenting various pathological conditions. The values of the serum/plasma prothrombin activity and the serum/plasma labile factor activity ratio ran a fairly parallel course, indicating that the percentage of labile factor utilized during the coagulation is roughly proportional to that of prothrombin consumed. On the other hand, the labile factor activity of serum was in definite inverse proportion to the concentration of the accelerator developed in serum. These relationships were well in evidence in all cases

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studied. In patients with a normal coagulation mechanism we constantly found a low prothrombin and low labile factor activity but a high accelerator effect in serum. These findings were reversed for patients in which the coagulation mechanism was defective because of deficiency of thromboplastin, prothrombin or labile factor. Thus, high serum prothrombin and serum labile factor activity but low serum accelerator were found in patients with: (a) hemophilia or thrombocytopenia, where very little thromboplastin was available(2); and (b) liver dysfunction, where deficiency of prothrombin and labile factor were associated. Defective formation of thrombin suggests itself as a common denominator (see later). A special condition was represented by the hypoprothrombinemia due to dicumarol therapy. The little amount of prothrombin available was well utilized, as labile factor and thromboplastin were normal, but a comparatively small amount of labile factor appeared consumed and little accelerator effect developed in serum.

The observations that: (a) the amount of labile factor utilized during coagulation is directly proportional to that of prothrombin; and (b) little labile factor is utilized during coagulation when either prothrombin or thromboplastin are deficient, clearly suggest that prothrombin, thromboplastin and labile factor react among themselves to form thrombin in definite quantitative proportions. These findings complement the previous report by Quick and Stefanini(9) that, in the presence of excess thromboplastin and an optimum amount of calcium, the addition of increasing quantities of labile factor (in the form of diluted deprothrombinized rabbit plasma) to stored human oxalated plasma induced a progressively increasing consumption of prothrombin.

Less clear is the significance of the inverse relationship between utilization of prothrombin and labile factor during coagulation and development of serum accelerator. It has been previously reported that the latter agent derives from the labile factor through the action of thrombin(3). It may then be suggested that little accelerator is formed whenever the formation of thrombin is slow and incomplete. This interpretation is confirmed by the findings in dicumarol hypoprothrombinemia. In this case the utilization during clotting of the limited amount of prothrombin available is high, but, as only a little thrombin can be formed, a high percentage of labile factor will be left unconverted and little accelerator found in the serum.

Summary. The percentage of labile factor and prothrombin activities and accelerator effect found in serum after completion of the coagulation of blood are in inverse quantitative proportions. A very small percentage of labile factor and prothrombin activity can be found in sera of healthy subjects; in this case high values of accelerator are the rule. Low values of accelerator and a large percentage of prothrombin and labile factor activity are found whenever the formation of thrombin is deficient because of the depletion of either thromboplastin (hemophilia, thrombocytopenic purpura) or prothrombin and labile factor (liver dysfunction). In dicumarol hypoprothrombinemia the available prothrombin is well utilized during coagulation, but the percentage in serum of unconverted labile factor is high and that of the accelerator low.

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The Use of Cortisone and ACTH in Rheumatoid Disease in Swine. (17912)

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In swine there are naturally occurring disease conditions which are somewhat similar to the so-called rheumatic or rheumatoid diseases in the human. These conditions include skin and joint manifestations as well as lesions in the heart and other visceral organs. Since these disease manifestations in swine are similar to rheumatic or rheumatoid conditions in the human, it is possible that any progress toward the understanding of the disease processes in one species may help in understanding similar processes in the other species. The clinical manifestations of rheumatoid disease in swine are quite variable. In some acute cases the body temperature may be as high as 108° F. Some of these acute cases show surprisingly few other symptoms; and they sometimes make spectacular spontaneous recoveries. Frequently there is evidence of soreness or stiffness in the legs, suggesting arthralgia or myalgia. A severe shifting lameness is sometimes seen. Affected animals may be unable or unwilling to stand or walk because of painful joints or muscles.

The skin manifestations are sometimes striking, particularly in white or light-colored animals. There may be a circumscribed erythema or the reddening may be diffuse, especially on the underside of the body. Occasionally the reddening of the skin assumes a pattern similar to the erythema annulare rheumaticum of Lehndorff-Leiner sometimes seen in the human. Subcutaneous nodules sometimes occur. In the fully developed chronic stage the joint enlargements in the legs are often conspicuous. The joint enlargement is due mainly to fibrotic periarthritis. The enlargement of the carpal joint is usually symmetrical, giving that portion of the leg a spindle-shaped appearance. The tarsal joint usually shows more enlargement medially than it does laterally. Varying degrees of ankylosis occur. The ankylosis is uusually fibrous, although bony ankylosis does occasionally occur.

The gross lesions consist mainly of increased connective tissue in and near the joint capsule, together with hypertrophy, increased vascularization and hyperemia of the synovial villi. The hypertrophic villi may largely fill the joint cavity. A membrane or pannus sometimes develops over a portion of the articular surface. Frequently there is considerable deterioration of the bone adjacent to the joint, resulting in roughening and pitting of the articular surface. Considerable deformity of the joint may occur. The gross visceral lesions are sometimes conspicuous, consisting principally of fibrinous or fibrous changes on the serous surfaces. Frequently there are extensive, firm adhesions between the visceral and parietal layers of the pericardium. Pleuritic and peritoneal adhesions sometimes occur. Vegetative formations are occasionally found on and around the auriculoventricular valves, particularly the bicuspid valve. Similar formations may occur on the semilunar valves. Microscopic examination often reveals an extensive myocarditis or pancarditis.

Since the clinical and pathological features of the disease or diseases here described in swine resemble rheumatic or rheumatoid diseases in the human, it was thought that arthritic pigs may be suitable experimental animals for determining the effects of some of the newer antirheumatic treatments. Consequently, cortisone and ACTH have been used on a few cases of naturally occurring rheumatoid type of arthritis in swine.

One pig weighing about 150 lb and showing well marked symptoms was given intra-

* The authors express their appreciation to the Lilly Research Laboratories and to Dr. K. K. Chen for the generous supplies of cortisone and ACTH used in this work.

muscular injections of 100 mg of cortisone daily. At the end of 4 days of treatment there was definite improvement. The treatment was continued for 3 more days making a total of 7 days. Two days after treatment was discontinued the symptoms began to return. Six days later the animal was unable to rise without assistance. After the animal had gone 31 days without treatment and had not shown any appreciable improvement, treatment with ACTH was begun. Daily intramuscular injections of 40 mg were given for 5 days without any appreciable effect.

A second hog weighing about 200 lb was given 100 mg doses of cortisone daily for 6 days. Improvement was shown on the fourth and was quite definite on the fifth day after treatment was started. There was no well marked recurrence of symptoms in this animal during a period of 30 days after treatment was discontinued.

A third shoat and a fourth one, weighing about 80 lb each were treated daily with cortisone and ACTH, respectively, in the same doses as used in the foregoing trials. On the third day of treatment both animals showed definite improvement. The one being treated with ACTH was almost free of lameness. Improvement continued in both animals during six days of treatment. Three days after treatment was discontinued both animals showed a return of symptoms. On the fifth day after treatment was stopped, the animal which had been treated with cortisone showed more marked symptoms than at any time. Treatment of this animal with cortisone was then resumed and continued for 4 days. On the third day after treatment was resumed there was definite improvement. Nine days after this treatment was discontinued, the animal was again showing marked symptoms of stiffness and lameness. The other shoat which had been treated with ACTH was showing fairly well marked lameness and stiffness 20 days after treatment was stopped.

Summary. Diseased conditions occur in swine which bear considerable resemblance to rheumatic or rheumatoid diseases in the human. Some naturally affected swine showed definite improvement following treatment with cortisone and also with ACTH. More work needs to be done before drawing conclusions as to how closely analogous are the rheumatoid conditions in swine and in the human.

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Mechanism of Increased Susceptibility to Ergot Gangrene in Thyrotoxicosis. (17913)

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Ergot gangrene in the human is still occasionally reported(1,2), but fortunately is now rare. It is usually attributable to too frequent or prolonged administration of large doses of ergot alkaloids, but occasionally follows small doses. It has often been reported in association with thyrotoxicosis(3), puerperal sepsis(4-7) and jaundice(8-11), but almost never following the use of these alka-

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TABLE I.
Influence of Alteration of Oxygen Consumption on Incidence of Ergotamine-Induced Gangrene in Rats.

Procedure	No. rats	Oxygen consumption (cc/min.) Avg	St. dev.	% change	Gangrene No.	%	p*	Significance
Propylthiouracil	16	2.12	.39	—41.3	8	50	>.20	0
Normal	26	3.61	.25	—	7	26.9	—	—
Thyroxin	15	5.43	.31	+50.4	15	100	<0.1	High

* Comparison of incidence of gangrene with normal.

loids in the treatment of migraine. This may be solely a question of the size of the dose employed for various conditions, but the impression has arisen that certain diseases predispose to ergot gangrene. Such a concept derives partial support from the observation that administration of thyroxin to rats increases the incidence of ergotamine induced gangrene(12). This observation is confirmed in the present study and evidence is presented that the effect is due to prolongation, by thyroxin, of the circulatory impairment produced by ergotamine.

Experimental. The incidence of gangrene of the tail was determined in a series of 26 normal rats weighing 140-200 g Following a single intraperitoneal injection of 12.5 mg/kg of ergotamine tartrate,* it was observed (Table I) that within 7 days, 7 or 26.9% of these animals developed gangrene of the tail. In order to determine whether thyrotoxicosis increased the incidence of gangrene, a series of 15 rats was injected intraperitoneally with 0.1 mg per day of crystalline thyroxin† for 10 days. During this period the oxygen consumption increased from 3.61 cc to 5.43 cc/min/animal, an average increase of 50.4%. Following this period the previous dose of ergotamine (12.5 mg/kg) was injected intraperitoneally.

Gangrene occurred in 100% of this group of rats, a significant increase in the incidence. All of these animals developed gangrene within 48 hours of the injection of ergotamine, whereas, only approximately 7% of normal animals which get gangrene from ergotamine do so within 48 hours(13). We can thus confirm the observation that thyrotoxicosis increases the susceptibility of the rat to ergotamine-induced gangrene and contribute the additional observation that gangrene occurs earlier in such animals. In contrast to previous observation(12), we find no increase in the lethal action of ergotamine. All of our thyrotoxic animals survived a dose of ergotamine fatal to 13.3% of normal animals.

Gangrene occurs as a result of vascular occlusion due to prolonged severe vasospasm produced by ergotamine. An increase in the incidence of ergotamine induced gangrene, as produced by thyroxin, might be due to either an increase in severity of the vascular occlusion or a decrease in the resistance of the tissues to vascular occlusion.

If the increased incidence of gangrene produced by thyroxin is due to increased tissue susceptibility to vascular occlusion on the basis of increased oxygen requirements, then a decrease in oxygen consumption should reduce the incidence of gangrene. Hence, 16 rats were given 25 mg per day by mouth of propylthiouracil for 6-8 weeks. At the end of this period the average oxygen consumption was 2.12 cc/min/animal, a decrease from normal of 41.3%. At the end of this period these animals were injected with 12.5 mg/kg of ergotamine. Eight or 50% of these animals developed gangrene and did so within

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* Gynergen-Sandoz 1:1000 solution.

† Furnished us through the courtesy of Dr. K. W. Thompson of Roche-Organon.

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TABLE II.
Influence of Thyroxin on Ergotamine Induced Restriction of Bleeding Volume in Rats.

Hr after inj. of ergotamine	No. of rats		Bleeding volume					
			Normal		Thyroxin treated			
	Normal	Thyroxin treated	Avg	St. dev.	Avg	St. dev.	p*	Significance
Control	11	8	8.43	3.02	8.86	2.93	>.50	None
4	19	14	1.05	1.52	1.59	1.69	>.30	"
10	22	16	2.86	2.15	2.77	3.03	>.50	"
16	19	13	4.24	3.16	1.67	1.55	.01	High

* Comparison of bleeding volume of thyroxin-treated animals with those of controls.

7 days of injection. The incidence of gangrene in these animals (Table I) is not significantly different from that of the controls. Thus, it is assumed that the mechanism of the increased incidence of ergot gangrene produced by thyroxin is not altered tissue susceptibility on the basis of increased tissue requirement for oxygen, but an increase in the degree or duration of the vascular occlusion produced by ergotamine in the presence of thyrotoxicosis.

We have previously reported(13) that within 1 hour of the injection of gangrene producing doses of ergotamine in the rat, the tail may be amputated within 3 cm of the body with no appreciable bleeding. The amount of bleeding from the amputated tail provides a basis for the rough measurement of the degree and duration of vascular impairment produced by ergotamine in normal and thyrotoxic rats. Seventy-one normal rats were injected intraperitoneally with 30 mg/kg of pentobarbital sodium and sufficient heparin (2-3 mg) to prevent clotting of blood. At varying periods following the intraperitoneal injection of 12.5 mg/kg of ergotamine tartrate, the tails were amputated 3 cm from the base with sharp rib shears and the blood collected in graduated centrifuge tubes. Care was taken to continuously remove gross clots appearing on the amputated stump. The bleeding volume was recorded 2 hours following amputation, at which time bleeding from all animals had stopped.

The average bleeding volume in 11 normal rats (Table II) prior to the injection of ergotamine was 8.43 cc. Four hours after injection of ergotamine, the average bleeding volume in 19 rats was reduced to 1.05 cc. Eight of these animals did not bleed at all from the amputated tail in 2 hours. The average bleeding volume progressively increases with time, becoming 2.86 cc at 10 hours and 4.24 cc at 16 hours. However, at 16 hours the bleeding volume is still significantly less ($p = <0.01$) than the pre-injection volume. It is only approximately 50% of this value. Thus, doses of ergotamine which may in a single injection cause gangrene, induce severe and prolonged spasm of the major arteries of the tail.

To test the effect of thyroxin on this vascular spasm, 51 rats were injected intraperitoneally with 0.1 mg/day of crystalline thyroxin for 10 days. They were prepared as were the control animals and the tails were amputated at the same time intervals. The average bleeding volume of the thyroxin treated animals (Table II) prior to the injection of ergotamine was 8.86 cc, not significantly different from the normal controls. Consequently, the effect of thyroxin on gangrene due to ergotamine is not due to an independent action of thyroxin on the vascular system. The average bleeding volume 4 hours after the injection of ergotamine was greatly reduced (1.59 cc), but not significantly more than the corresponding normal animals 4 hours after ergotamine injection. At its height, the ergotamine induced vasospasm is not greater in thyroxin treated than in normal animals. Hence, the potentiating effect of thyroxin would not seem to be an increase in the severity of the vasospasm. Correspondingly, the bleeding volume is not significantly different in the thyroxin treated group from that of the normal 10 hours after the injection of ergotamine. However, 16

hours after injection the average bleeding volume in the thyroxin treated animals (1.67 cc) is significantly less than that of the normal animals (4.24 cc) and is only approximately 18% of the value obtained prior to the injection of ergotamine.

It appears that the effect of thyroxin in increasing the susceptibility of the rat to ergotamine-induced gangrene of the tail is essentially one of prolongation of the vascular occlusion produced by ergotamine. Such an effect may be due to sensitization of the vascular musculature to the action of ergot as has been claimed for epinephrine(14). It would seem that such should result in an increase in the intensity as well as the duration of the effect. However, the present method may be too indelicate to detect an increase in an already severe vasospasm. Alternatively, thyroxin may interfere with the destruction and/or elimination of the ergot alkaloids, thus, prolonging without intensifying the effect.

In partial support of this latter hypothesis is the observation that 8 out of 10 rats developed gangrene following the injection of 12.5 mg/kg of ergotamine subsequent to exposure to chloroform vapor (2 hours in a concentration of 1.77 vol. %). This is a significant ($p = <0.02$) increase in the incidence of gangrene over normal and suggests that liver damage, by interfering with the elimination of ergot alkaloids, may predispose to ergot gangrene.

Summary. Thyrotoxicosis increases the incidence of ergotamine-induced gangrene of the tail of the rat. Reduction in oxygen consumption by propylthiouracil does not alter the incidence of ergot gangrene. Bleeding from the amputated tail of the rat is markedly reduced for prolonged periods by ergotamine and pre-treatment with thyroxin further prolongs the vasospasm so induced. It is assumed that thyroxin acts, not by altering the resistance of the tissues to vascular occlusion, nor by sensitizing the vascular musculature to ergotamine, but by prolonging the action of the alkaloids, possibly by interfering with their elimination or destruction.

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Effect of Hyperglycemic-Glycogenolytic Factor on Blood Sugar of Patients with Diabetes. (17914)

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In a previous communication(1) the clinical and experimental evidence suggesting that a hyperglycemic substance obtained from the pancreas may be an alpha cell hormone which causes an elevation of blood sugar by promoting hepatic glycogenolysis was reviewed. As stressed by Weisberg *et al.*(2) thus far it has not been proved that this material functions physiologically nor that it is a true hormone. This substance has been designated by Sutherland and his co-workers(3-5) as a hyperglycemic-glycogenolytic factor or H-G F.

If the H-G factor is an alpha cell hormone, it was believed that certain patients might exhibit evidences of increased or decreased activity of this substance as suggested by Thorogood and Zimmerman(6), McQuarrie

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et al.(7) and others. Furthermore the possibility was entertained that variations in the activity of this factor might account for certain differences noted in patients with clinical diabetes. If the patient with "stable diabetes" has normal amounts of alpha cell function the administration of insulin might, directly or indirectly, produce a secretion of the H-G F preventing hypoglycemia and possibly increasing the insulin requirement. Some patients with a labile form of diabetes might have inadequate amounts of the H-G F and would therefore be very sensitive to insulin and show greater fluctuations of blood sugar after food or insulin. Such a patient might also develop acidosis readily since it has been suggested(6) that the alpha cell hormone might prevent or decrease ketosis. Patients in this group might also respond more vigorously than the stable diabetic when the H-G factor is administered. Hildes, Sherlock and Walsh(8-9) studied hepatic glycogen stores in diabetic patients and found them to be somewhat low but within the range of normal values. They also measured the responsiveness of different patients to a constant infusion of adrenalin. They noted that certain patients with labile diabetes failed to show a normal rise of blood sugar and some of these same patients exhibited a rise in hepatic glycogen levels in contrast to the normal and the stable diabetic group. This might suggest that these individuals are unable to use their hepatic glycogen stores in a normal manner and that they may be lacking in some factor necessary for adequate glycogenolysis.

The intravenous injection of insulin for determining insulin sensitivity and in treating patients in diabetic coma has not been attended by any remarkable or unpredictable side effects. It was felt that insulin preparations treated so as to destroy the insulin content but permit the continued activity of the H-G factor, which is present as a contaminant, should have no deleterious effects. Material thus obtained was injected into mice subcutaneously and intraperitoneally. It has been administered intravenously to rats, rabbits and dogs repeatedly and in large amounts with no evidence of toxicity. Three patients with inoperable carcinoma and one infant with hydrocephalus likewise exhibited no deleterious effects after receiving this substance in amounts as great as 1.0 cc per 10 kg of body weight. In view of the absence of any permanent or lasting effects beyond the temporary elevation of blood sugar the study to be reported was undertaken.

Material. Patients with diabetes were divided into 2 classes insofar as possible. The clinical classification suggested by Himsworth(10) was used although the insulin-glucose test devised by that author was not employed. One group consisted of older diabetics with blood sugar levels and glycosuria controlled by diet alone or in conjunction with varying amounts of insulin, in some instances as much as 100 units daily. These patients had rare episodes of hypoglycemia or acidosis, tended to be obese or normal in weight and for our purposes shall be referred to as "stable diabetics." A second group of diabetic patients were either young or had, with one exception (case No. 22), developed diabetes in early life. Their insulin requirement varied but in no instance was as high as some of the patients in the first group; they had experienced repeated episodes of hypoglycemia and of acidosis, even coma in some cases. These patients were thin or of normal weight. Control of the diabetes in this group was difficult to maintain. We shall consider these as "labile diabetics." A group of patients with diabetes which we were unable to classify,* with hepatic disease and a few patients with miscellaneous conditions were studied, as were a few patients convalescing from various diseases not involving disturbances of the liver or of carbohydrate metabolism.

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Methods. Two lots of Insulin, Squibb (No. 886 and 888) containing 80 units per cc and known to have relatively large amounts of the H-G factor were treated by heat and alkali as described by Sutherland(3). To 10 cc of the insulin preparation were added 0.8 cc of normal sodium hydroxide. This mixture was then incubated for 3 hours at 37°C. At the end of this time 0.8 cc of normal hydrochloric acid was added. The material was cultured for sterility and 1.0 cc injected subcutaneously into a mouse, to test for residual insulin. Patients were tested by injecting intravenously 0.5 cc of the "inactivated insulin" preparation per 10 kg of body weight. Some tests were performed in the fasting state in the early morning and others in the late morning after the patient had received breakfast and the usual morning dose of insulin. Blood sugar determinations were made by the method of Folin and Wu (11) on specimens taken prior to the injection and at 15 and 30 minute intervals. In a number of cases, as well as in many experimental observations in animals, specimens were also obtained at 5, 45, 60, 90, and 120 minutes. Little added information was thus obtained. In a few of these cases adrenalin tolerance tests were performed as described by Kinsell(12). These tests were performed on the same patients as were the tests with the H-G factor and under conditions as nearly identical as possible with those employed in the former test. Adrenalin chloride was injected subcutaneously, 0.1 cc of a 1:1000 solution per 10 kg of body weight being administered. Blood specimens were obtained prior to injection and at 30, 45, and 60 minute intervals for sugar determination.

Results. Systemic reactions consisting of urticaria, erythema or pruritis occurred in 3 patients, 2 of whom had in the past received insulin but did not require it when the test was performed. In 1 additional case a strongly positive skin reaction precluded the performance of the test. The reactions responded readily to antihistaminic preparations and adrenalin. Repeated tests performed on 3 patients before breakfast and in the late morning with different initial sugar levels in 2 showed a rise of the same magnitude whether the initial level was high or relatively normal. Therefore, the actual rise in blood sugar in milligrams per 100 cc has been presented rather than the percentile change. Experimental observations in animals also suggested that the initial blood sugar levels did not affect the elevation of blood sugar. The results obtained in this group of studies are presented in Table I. It will be noted that a fall in blood sugar occurred in several instances. We are not able to account for these cases but minimal amounts of insulin may have been present in some preparations. The normal patients and patients with a stable form of diabetes showed rises of blood sugar which were quite comparable, averaging 13.2 and 17.5 mg per 100 cc respectively with a maximum rise in this group of 25 mg per 100 cc. Among the labile diabetics, 6 tests performed in 5 patients produced an elevation of 41 to 108 mg per 100 cc. A sixth patient (Case No. 23) with diabetes which must be classified as labile had mild hypoglycemic symptoms and low blood sugar levels. When tested, at noon, with both adrenalin and the H-G factor she failed to respond to either of these agents. The average rise for this entire group was 57.7 mg† per 100 cc.

Too few adrenalin tests were performed to draw any conclusions. There was no evident correlation, however, between the rise of the blood sugar in any individual case

* Case No. 24, 25, 26 have at times required or at least tolerated large doses of insulin, (80, 150, and 65 units daily) at other times have been well maintained on no insulin. Case 27 is a patient with diabetes and recent Addison's disease. Case 28 has had repeated episodes of pancreatitis with calcification of the pancreas, steatorrhea and diabetes requiring 15 units of insulin daily in the past.

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† Statistical analysis of these data reveals a "t" value of 13.5. From the probability tables it would appear that the possibility of these results occurring as a matter of chance would be considerably less than 1%.

TABLE I.
Effect of Intravenous Injection of Hyperglycemic-glycogenolytic Factor and Subcutaneous Injection of Adrenalin on Blood Sugar Levels of Patients.

					Hyperglycemic factor from inact. insulin			Adrenalin		
Case No.	Sex	Age, yrs	Wt., lb.	Insulin, U/day	Initial bl. sugar mg %	Max. change bl. s., mg %	Time, min.	Initial bl. sugar, mg %	Max. change bl. s., mg %	Time, min.
					Normal					
1	M	21	140	0	95	21	(15)	98	77	(45)
2	M	23	152	0	97	15	(15)	94	83	(45)
3	M	28	145	0	84	19	(15)			
4	M	24	160	0	80	20	(15)			
5	M	31	168	0	121	—9	(30)			
					Stable diabetics					
6	M	42	151	25	100	14	(5)			
7	M	58	170	15	133	10	(15)			
8	M	49	185	20	139	19	(15)			
9	M	46	125	24	272	26	(30)			
9				20	141	20	(15)			
10	M	61	185	30	121	24	(15)			
11	M	49	190	15	80	20	(15)			
12	F	38	150	55	226	22	(15)	232	17	(45)
13	M	48	175	0	99	25	(30)	102	98	(60)
14	F	45	161	30	177	22	(15)	248	—14	(45)
15	F	58	218	100	356	18	(15)			
16	F	42	243	105	324	—8	(15)			
17*	F	65	154	35	300	16	(15)			
					Labile diabetics					
18	M	18	127	40	225	42	(15)			
19	M	54	160	40	140	60	(45)			
19		55	158	56	250	75	(30)	179	169	(60)
20	M	19	162	75	127	41	(15)			
21	M	19	133	60	266	108	(15)	324	22	(30)
22	F	50	115	35	438	74	(30)	330	42	(45)
23	F	22	129	52	40	4	(15)	58	3	(30)
					Unclassified diabetics					
24	M	24	145	0	232	3	(15)			
25*	F	46	166	0	114	10	(15)	118	49	(60)
26	M	54	137	0	62	22	(15)			
27	F	48	137	0	119	26	(15)	98	35	(45)
28*	M	39	132	0	127	31	(15)	167	95	(60)
					Hepatic diseases					
29	F	42	122	0	204	—75	(60)			
30	M	49	142	0	91	43	(15)	94	92	(45)
31	F	42	137	0	80	15	(15)			
32	F	50	177	0	110	6	(15)			
32		51	180	0	97	13	(15)	87	63	(45)
					Miscellaneous					
33	F	2.5	32	0	23	11	(15)	30	8	(45)
34	M	48	170	0	99	14	(15)			

* Reaction occurred soon after the injection of the HGF.

in response to either the H-G factor or adrenalin.

Comment. It would seem that if the labile diabetic is deficient in alpha cell function, a certain amount of glycogen deposited in the liver is not used as adequately as in the normal individual. When the H-G factor is administered, even in small amounts, this liver glycogen is mobilized. Lukens(13) suggested that the H-G factor, as it is con-

cerned in carbohydrate metabolism assists, rather than opposes, the action of insulin since it makes hepatic glycogen stores available as blood sugar for the insulin activity and further metabolism. The presence of this hypothetic additional hormone may account for some of the metabolic discrepancies heretofore encountered in clinical diabetes.

It should be stressed again that no conclusive proof exists that the alpha cell produces the H-G F nor that this is actually a hormone. It is possible, certainly, that this substance produces its effect in these cases because of some abnormality in the hepatic glycogen formation or storage permitting different glycogenolytic agents to act differently. Therefore, the possibility of treating these young labile diabetic patients with the H-G factor in an effort to make their diabetes easier to control, even though larger doses of insulin would then be required, is worth some speculation but such efforts should await further study of other metabolic implications which will be discussed elsewhere.

Conclusions. A small number of patients were treated with the H-G factor obtained as a contaminant of certain insulin preparations. Five of 6 patients with labile diabetes showed a rise in blood sugar greater than was seen in any of the normal subjects or stable diabetic patients.

I am indebted to Miss J. Hurwitz for technical assistance and to Mr. G. Blair and Dr. L. Hines of E. R. Squibb and Company for generous supplies of insulin.

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Ultraviolet Photography of Paper Chromatograms in the Study of Nucleic Acids.* (17915)

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In the course of studying, by paper chromatography, the enzymatic degradation of DNA and RNA by streptococcal enzymes, a simple method of photographing the paper strips in the ultraviolet was developed, and forms the basis of this report.

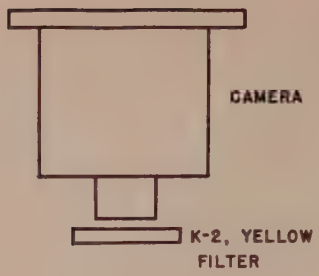
Method. Fig. 1 illustrates the mechanical system employed. The ultraviolet light source was a model SL Mineralight† containing a 2537 short wave filter. The paper chromatogram was fastened tautly across an open box or taped to the undersurface of a supported sheet of clear glass (*i.e.*, with the paper facing the light source). Any camera capable of focusing at close distances was found suitable when used with a light yellow filter (*e.g.*, K_2 filter). High contrast film (*e.g.*, Process Pan) was preferable, although overdevelopment of ordinary film produced satisfactory contrast. A distance of 14 inches from light source to paper, and an exposure time of one hour at F 4.5, using a film with a Weston rating of 48, gave consistently satisfactory results. Further distances from the light source gave more even illumination of the paper, but made longer exposure times necessary. With the lighting source described, the method of Markham and Smith(1), which employ contact printing, could be simplified, but was not found to be as convenient in our enzymatic studies.

Results. Fig. 2 illustrates an ultraviolet photograph, by the procedure outlined, of a paper chromatogram of 1% DNA and RNA

* This study was supported by a grant from the National Institute of Health, United States Public Health Service.

† Ultraviolet Products, Inc., South Pasadena, Calif.

1. Markham, R., and Smith, J. D., *Biochem. J.*, 1949, v45, 294.



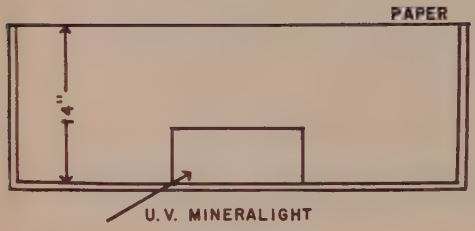


FIG. 1.
Schema of method for ultraviolet photography.

hydrolysates. Hydrolysis of the nucleic acids was carried out with perchloric acid as described by Marshak and Vogel(2). Ten μl of the hydrolysate was deposited at the starting point and a N-butanol: IN ammonia (7:1) solvent system employed. The 4 bases derived by hydrolysis from each type of nucleic acid and the solvent front are readily visualized. Adenine and guanine have very characteristic appearances in the presence of perchlorate. Note that the photography demonstrates that uracil is distinct from the adenine crescent in the RNA hydrolysate with this solvent system. This is not apparent when the paper is scanned with an ultraviolet lamp in the conventional manner.

The advantages of this photographic procedure in the study of nucleic acid by paper chromatography are apparent. Permanent objective records are obtained for careful study, filing, characterization of each of the spots, and extremely accurate measurement of RF values. After being photographed, the spots on the paper can be eluted in the conventional manner, and further identification made. The sensitivity of the photographic method is such that amounts of material too small to estimate accurately by the spectrophotometer can be readily visualized.

Summary. A simple method for the ultraviolet photography of paper chromatograms has been described, and its advantages in the study of nucleic acids noted.

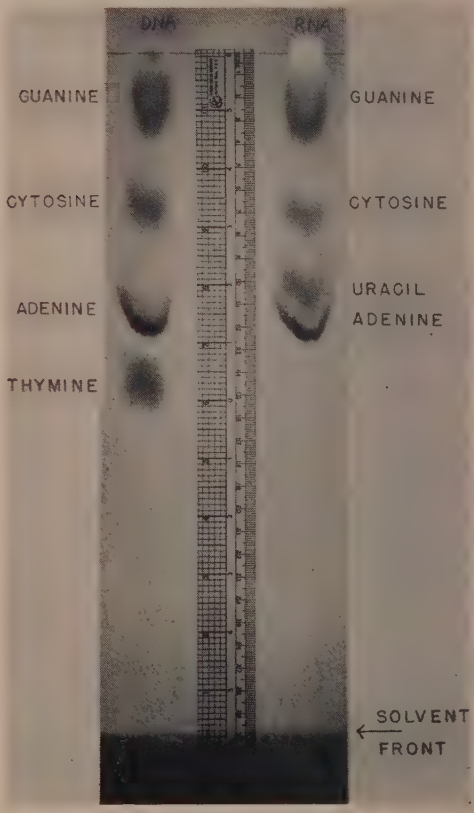


FIG. 2.
Ultraviolet photograph of paper chromatogram of a 1% DNA and RNA hydrolysate. Photo taken with a 4 x 5 Speed Graphic, 135 mm Optar lens, K_2 filter, exposure 1 hr at F 4.5 on Kodak Process Pan.

2. Marshak, A., and Vogel, H. J., *Fed Proc.*, 1950, v9, 85.

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Amino Acids of Nervous Tissue. (17916)

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In previous work patterns of free amino acids were determined by paper chromatography in various normal mouse tissues and in tumors(1,2). Each normal tissue examined had a distribution characteristic of that tissue, while all of the tumors, regardless of derivation or source, showed similar patterns. Schurr and co-workers(3), using microbiological procedures, have reported differing patterns of free amino acids in the plasma, brain, liver, spleen, and muscle in the rat, but they did not make determinations of glutamic acid, aspartic acid, glutamine, taurine, glycine, alanine, serine, and cystine, the amino acids found in our previous studies to be present in the highest concentrations in the free form in mouse tissues. They were able to account for only approximately 7% of the free α-amino nitrogen of brain. It was therefore of interest to report our observations on the distribution of those amino acids which are present in nervous tissue in the highest concentrations and which undoubtedly account for the greatest proportion of the free amino nitrogen.

Experimental. Animals and preparation of tissues. All mice employed in the present study were killed by dislocation of the cervical vertebrae. The rabbits were usually killed in the same way or deeply anesthetized with intraperitoneal nembutal. Similar tissue removed after either procedure yielded identical results. Whole mouse brains were used. In the case of the rabbits samples were taken from the cerebrum (cerebral cortex), cerebellum, and upper brain stem and samples of sciatic nerve and the lumbosacral spinal cord were also studied. The extracts of the tissues were prepared for analysis essentially as previously described(1). Aliquots corresponding to 75 mg of fresh tissue were employed. The alcoholic extracts gave the same results as dialysates prepared from heat coagulated tissue. The mouse brains were from animals of the C3H, $C57_6$, dba, and Swiss strains. The neuroblastoma was the transplanted tumor C1300 carried in strain A mice at the Jackson Memorial Laboratory. Most of the rabbits used were of the New Zealand White Stock. The ataxic rabbit was of the Sandy Flemish strain. Part of the lumbosacral spinal cord of one of the rabbits was removed 6 days after section of both sciatic nerves.

Chromatography. Suitable volumes of the samples were placed on paper for 2-dimensional paper chromatography by the method of Consden, Gordon, and Martin(4). Phenol saturated with water was used as the first solvent and a mixture of collidine and lutidine saturated with water as the second. The spots were treated with hydrogen peroxide (5) prior to chromatography in a number of instances to convert methionine to the sulfone and cystine to cysteic acid.

Results. Comparison of pattern of free amino acids with that of hydrolyzate of whole brain. A chromatogram made of an acid hydrolyzate corresponding to 10 mg of fresh whole mouse brain (Swiss) is shown in Fig. 1. In addition to the amino acids ordinarily found in proteins, β-alanine, ethanolamine, glucosamine, α-aminobutyric acid and γ-aminobutyric acid were observed. Phenylalanine was poorly separated from the leucines be-

* Aided by grants from the American Cancer Society and Charles F. Kettering Foundation.

1. Roberts, E., and Frankel, S., *Cancer Research,* 1949, v9, 645.

2. Roberts, E., and Frankel, S., *Cancer Research,* 1950, v10, 237.

3. Schurr, P. E., Thompson, H. T., Henderson, L. M., Williams, J. N., and Elvehjem, C. A., *J. Biol. Chem.,* 1950, v182, 39.

4. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.,* 1944, v38, 224.

5. Dent, C. E., *Biochem. J.,* 1947, v41, 240.

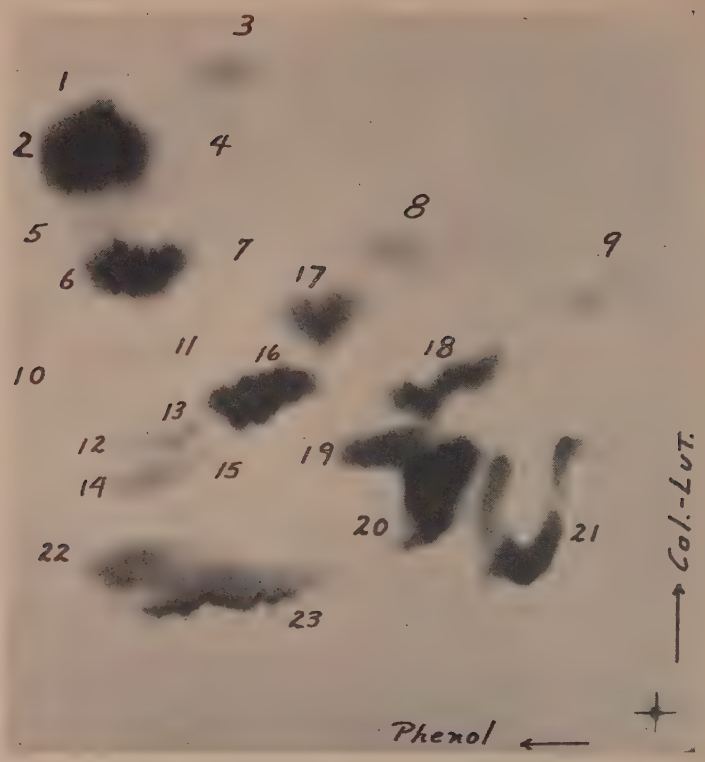

FIG. 1.
Chromatogram of hydrolyzate of 10 mg of whole mouse brain.

Key to numbers on chromatograms: phenylalanine, 1; leucine and isoleucine, 2; tyrosine, 3; glucosamine, 4; ethanolamine, 5; valine, 6; methionine (methionine sulfone), 7; taurine, 8; cystine (cysteic acid), 9; proline, 10; α-amino-N-butyric acid, 11; histidine, 12; hydroxyproline, 13; γ-aminobutyric acid, 14; β-alanine, 15; alanine, 16; threonine, 17; serine, 18; glycine, 19; glutamic acid, 20; aspartic acid, 21; arginine, 22; lysine, 23; glutamine, 24; glutathione, 25; X and X^1 are unknown ninhydrin-reactive substances which disappear on acid hydrolysis; X^{11} and X^{111} are also unknown substances.

cause of the large amounts of the latter. Tryptophan was not detected because it is destroyed during acid hydrolysis. Fig. 2 shows a typical chromatogram of the free amino acids extracted from 75 mg of brain. It is apparent that the pattern does not reflect the amino acid composition of the brain as a whole. A number of the amino acids found in large quantities in the hydrolyzate were present in the free form in amounts below the limit detectable by the chromatographic procedure. Glutamic acid, taurine, aspartic acid, and γ-aminobutyric acid were present in the largest amounts in the free form. The identity of the γ-aminobutyric acid, found at most in traces in tissues other than brain, has been established unequivocally(6,7). Somewhat smaller amounts of cystine, serine, glycine, alanine, and glutamine were found. Free valine, the leucines, threonine, glutathione and β-alanine were detected. On some chromatograms the leucine spot was not found. In addition, two ninhydrin-reactive substances designated x and

6. Roberts, E., and Frankel, S., *Fed. Proc.*, 1950, v9, 219.

7. Udenfriend, S., *Fed. Proc.*, 1950, v9, 240.

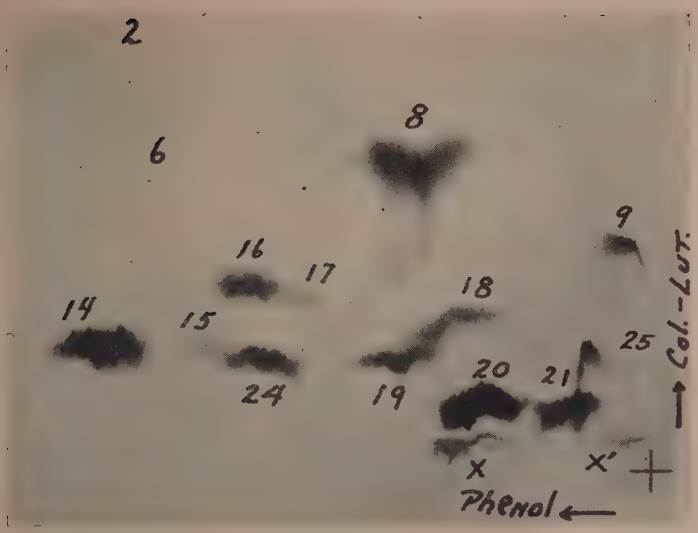

FIG. 2.
Extract of 75 mg of adult mouse brain.

x^1 which disappeared on acid hydrolysis were located below aspartic and glutamic acid on the chromatograms. Some constituents may have been present in quantities below the detectable range. The results of Schurr *et al.*(3) obtained by the more sensitive microbiological methods indicate that probably all of the amino acids found in cellular proteins are present to some extent in tissues in the free form.

Influence of age, sex, and strain on free amino acids of mouse brain. Chromatograms were made of extracts of the brains of mice immediately after birth and one day before birth and were compared with those obtained from the brains of the mother mice. The fetal brains had less glutamic acid and aspartic acid than the brain of the mother, and more taurine, alanine and substance x. Small amounts of arginine, tyrosine, and α-aminobutyric acid were found in the fetal brain, but were not detected in the adult brain. The other constituents were noted in approximately the same amounts in both samples. Extracts of the brains of the newborn mice had less γ-aminobutyric acid, glutamic acid, cystine, and aspartic acid and more taurine and material under glutamic acid than did the extract of the maternal brain. One pooled sample of brain obtained from a litter of 15-day embryos showed no γ-aminobutyric acid and only a trace of glutamine. However, there were considerably larger quantities of glutathione and the intensity of spot x was much greater in the embryonic brain. An examination of the brains of male and female littermate mice (30-37 days old) of the C3H, $C57_6$, and dba strains revealed no essential differences between the sexes. The general impression was obtained that the quantity of detectable amino acids decreased in the order C3H, $C57_6$, dba. However, this will have to be checked by quantitative procedures.

Free amino acids in tumor C1300 (neuroblastoma). Although no direct comparison can be made between the transplantable neuroblastoma and any of the samples of mouse brain examined, it was, nevertheless, of interest to study this tumor, which was the only malignancy of nervous origin available to us. The pattern of free amino acids consistently observed in a number of samples of this tumor was similar to that found for other transplantable mouse tumors(1) and different from that found in the typical brain chromatogram (Fig. 3). The pattern of the

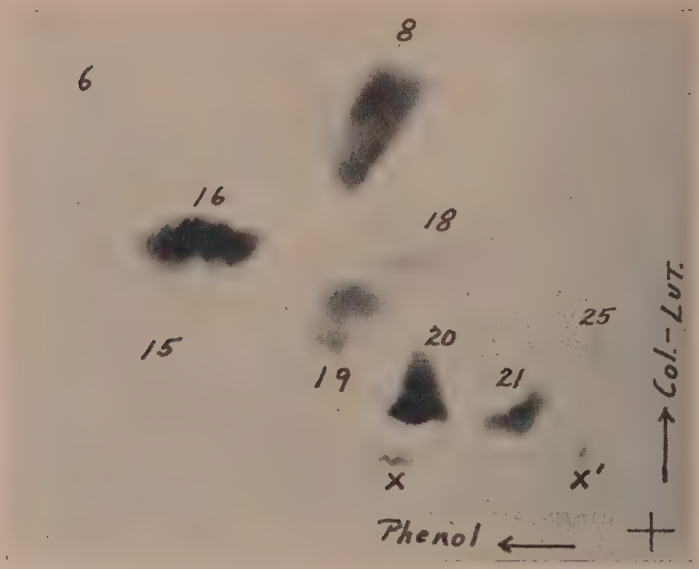

FIG. 3.
Extract of 75 mg of neuroblastoma (tumor C1300).

neuroblastoma was very similar to that of the 15-day embryonic brain, showing no glutamine or γ-aminobutyric acid.

Free amino acids in rabbit nervous tissue. Cerebrum (cerebral cortex) upper brain stem, spinal cord, and sciatic nerve were studied in normal rabbits and in an ataxic rabbit. The distributions of free amino acids in the brain were essentially similar to those found in mouse brains, but the taurine levels of the different parts of the rabbit brain were much lower. Somewhat smaller amounts of some of the constituents were noted in the spinal cord. The sciatic nerve showed much smaller concentrations of most of the detectable constituents and no γ-aminobutyric acid. Relatively large quantities of the latter substance have been observed in every sample of mouse brain and rabbit brain and spinal cord examined. Fig. 4 shows a typical chromatogram for cerebrum (cerebral cortex). The differences in free amino acids of the sciatic nerve and the various parts of the central nervous system may reflect genuine metabolic differences or may be the result of smaller amounts of neuroplasm per unit of fresh weight in the sciatic nerve. The concentrations of free amino acids in the nervous tissues of the moribund ataxic rabbit were much lower than in the corresponding tissues of the normal rabbits. No changes in the distribution of free amino acids in the lumbosacral portion of the spinal cord of the rabbit were noted 6 days after section of both sciatic nerves. It is interesting that in this species relatively little histological change occurs in the spinal cord after severing the sciatic nerves(8).

Homogenate experiments. The high contents of free glutamic and aspartic acids, glutamine, and alanine in brain are consistent with the important roles that these substances play in nitrogen transport in the cell (8). A number of experiments, which will be reported in detail elsewhere, were performed in which homogenates of brain were incubated in 0.05 M phosphate buffer (pH 7.4) at 38°C in an atmosphere of oxygen, with or without added glutamic or aspartic

8. Bodian, D., and Mellors, R. C., *J. Exp. Med.*, 1945, v81, 469.

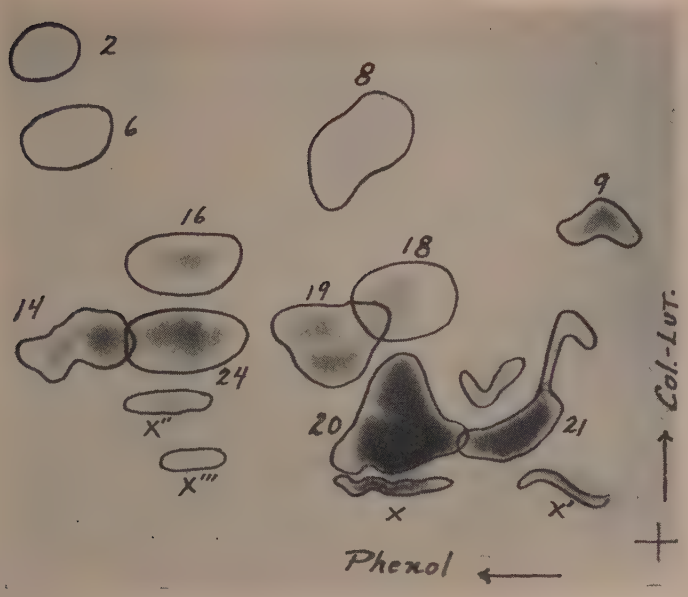

FIG. 4.
Extract of 75 mg of rabbit cerebrum (cerebral cortex).

acids. Suitable aliquots of protein-free extracts of the incubation mixtures were subjected to chromatography. The results clearly showed the presence of enzymatic mechanisms both for the breakdown and synthesis of glutamine. The existence of transaminating mechanisms was shown by the formation of alanine and aspartic acid in the presence of added glutamic acid, and by the increases in the contents of alanine and glutamic acid when aspartic acid was the substrate. It is well known that brain tissue can effect transamination (9) and synthesize glutamine from glutamic acid (10). In addition, γ-aminobutyric acid was found to be formed at an appreciable rate in the presence of added glutamic acid and removed in the presence of added aspartic acid. The latter findings are being investigated further. No notable changes were observed in the contents of amino acids other than those mentioned above under the conditions of the experiments.

Summary. The pattern of free amino acids in mouse brain, as shown by 2-dimensional paper chromatography, does not reflect the amino acid composition of the brain as a whole. The brains of mice of several strains and various parts of the nervous system of the rabbit were studied. Free glutamic and aspartic acids, γ-aminobutyric acid, taurine, glutamine, cystine, serine, glycine, alanine, valine, and the leucines were detected consistently. The variations in these constituents with age, sex, and strain in mice are discussed. The sciatic nerve in the rabbit had considerably smaller quantities of detectable constituents than the various parts of the central nervous system. A transplantable neuroblastoma in mice had a pattern of free amino acids which was similar to that found in other malignant tissues and in brains of 15-day embryos and significantly different from that found in all of the other samples of mouse brain examined. The suitability of the chromatographic method for the study of changes in the free amino acids of brain homogenates is indicated.

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Protective Effect of Fibrinogen on Accelerator-Globulin. (17917)

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Data has been obtained which indicates that the presence of fibrinogen may play an important rôle, not only in protecting accelerator-globulin (Ac-globulin) against various chemical and physical agents, but also, in inhibiting Ac-globulin activity in the conversion phase of the normal clotting process. In a previous study(1), oxalated bovine plasma was subjected to the action of chemical and physical agents which involved denaturation, adsorption, and precipitation phenomena. It was observed repeatedly that the amount of Ac-globulin and prothrombin removed or inactivated, in large part, depended upon whether or not the plasma had been defibrinated initially. Almost invariably, agents which effected a reduction of Ac-globulin activity and prothrombin concentration in undefibrinated plasma, effected a decidedly greater reduction in defibrinated plasma. Also, when precipitated fibrinogen was removed from plasma which had been frozen and subsequently thawed, Ac-globulin activity was significantly greater than that in fresh unfrozen plasma. Preliminary quantitative data suggest that there is probably an inverse relationship, within limits, between Ac-globulin activity and the amount of fibrinogen in plasma.

Methods. The source of prothrombin and an outline of the method used in the quantitative assay of Ac-globulin have been described elsewhere(1). Prothrombin concentrations were determined by the modified 2 stage technic(2,3). The thromboplastin and fibrinogen preparations were of the same type as those used previously(1). Parke-Davis topical thrombin was used exclusively. Beef, dog, and rat blood was collected in 1.85% potassium oxalate; human blood was collected in 2.85% sodium citrate; 7 parts of blood to 1 part of anticoagulant were used.

Observations. An example of the protective action of fibrinogen on Ac-globulin and prothrombin, and one which may be taken as representative of an adsorption phenomenon, is the treatment of plasma with kaolin. (Table I) When 5 g of kaolin was added to 100 ml of non-defibrinated bovine plasma, only 30% of the prothrombin and Ac-globulin was adsorbed. In contrast, when 100 ml of plasma was defibrinated with thrombin, and then treated with 5 g of kaolin (NF Powder), approximately 40-50% of the prothrombin and Ac-globulin was adsorbed. It required 17 g of kaolin per 100 ml of non-defibrinated plasma to adsorb 40-50% of the prothrombin and Ac-globulin. The removal or inactivation of Ac-globulin and prothrombin effected by the addition of protein precipitants of the heavy metal group is another instance in which the protective action of fibrinogen on Ac-globulin and prothrombin was demonstrated. In one experiment, when native bovine plasma was treated with an equal volume of a 1.5% $HgCl_2$ solution, centrifuged, and the mercuric ions removed with coagulated egg albumin, the Ac-globulin activity of the supernatant was approximately 10%, and the prothrombin concentration 35% of that in the native plasma. However, when defibrinated plasma was subjected to similar treatment, no Ac-globulin activity or prothrombin could be detected. Furthermore, the presence in the plasma of precipitated fibrinogen or of clumps of formed fibrin, which may result from freezing and subsequent thawing of the plasma, interferes with the removal or inactivation of Ac-globulin and prothrombin by $HgCl_2$ solution. When the precipitated fibrinogen and/or fibrin are removed from the plasma, how-

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2. Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, v114, 667.

3. Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.*, 1949, v19, 471.

TABLE I.
Effect of Kaolin on Prothrombin Concentration and Ac-Globulin Activity.

Physical state of plasma	% prothrombin conc.	% area	% Ac-G activity
1. Fresh native beef plasma	100	100	100
2. (1) treated with kaolin	69	92	70
3. (1) defibrinated with thrombin and treated with kaolin	58	61	51

The kaolin conc. was 5 g per 100 ml of plasma. Initially kaolin was mixed with distilled water, the mixture centrifuged, the supernatant decanted, and the tube inverted and allowed to drain. Beef plasma was added to kaolin, mixed, swirled for 5 min., centrifuged, and the clear supernatant plasma removed. For each Ac-globulin determination, .03 ml of plasma was used.

TABLE II.
Effect of Freezing on Ac-Globulin Activity.

Physical state of plasma	ml of plasma	% area	% Ac-G activity
1. Fresh pooled human plasma, 28°C	.01	100	100
1a. (1) stored at −40°C, 5 hr, thawed at 28°C; precipitated fibrinogen removed	.01	177	334
2. Fresh beef plasma, 28° C	.0009	100	100
2a. (2) same as (1a), stored 3 days	.0009	136	175
3. Fresh rat plasma, 28°C	.1	100	100
3a. (3), 28°C, spontaneous precipitation of fibrinogen; precipitate removed	.1	187	353
4. Fresh dog plasma, 28°C	.02	100	100
4a. (4) same as (1a), but stored 4 hr	.02	133	220
4b. Fibrinogen redissolved in dog plasma, 5°C	.02	107	125
5. Fresh dog plasma, 28°C	.002	100	100
5a. (5) same as (1a), but stored 24 hr; no precipitation of fibrinogen	.002	80	60
6. Fresh beef plasma, 28°C	.009	100	100
6a. (6) defibrinated with thrombin, 28°C	.009	141	182
6b. (6a) same as (5a), stored 3 hr	.009	144	185

ever, the same concentration of $HgCl_2$ solution effects complete removal or inactivation of both Ac-globulin and prothrombin. If fibrinogen does manifest some type of protective action toward Ac-globulin, as our evidence would suggest, it may, in an analogous manner, serve to inhibit Ac-globulin activity in the clotting process. This would provide a probable explanation for the observation that Ac-globulin activity is decidedly greater in plasma that has been frozen than in plasma that has not. The data in Table II, substantiate this observation in 4 species of animals. It should be noted that increased Ac-globulin activity occurs only when fibrinogen is precipitated out of the solution, either by freezing and subsequent thawing (1, 1a, 2, 2a, 4, 4a), or by spontaneous precipitation (3, 3a). When, in spite of freezing and thawing, no fibrinogen is precipitated, there is some decrease of Ac-globulin activity (5, 5a). When the fibrinogen is redissolved in the same plasma from which it was precipitated, Ac-globulin activity is reduced almost to that of fresh unfrozen plasma(4). We have been unable, however, to reduce the Ac-globulin activity of frozen plasma to that of unfrozen plasma by the addition of Armour bovine fibrinogen. There is no significant difference in Ac-globulin activity in frozen and unfrozen defibrinated plasma, but there is a decided difference in activity in defibrinated (with thrombin) and non-defibrinated plasma (6, 6a, 6b). There is only a slight difference in Ac-globulin activity in plasma defibrinated with thrombin (6a) and that defibrinated by freezing (2a), the latter, moreover, not being free entirely from fibrinogen. Prothrombin concentrations of the species tested are not altered detectably by the initial freezing and thawing of plasma.

Discussion. These studies serve to intro-

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duce the concept that fibrinogen apparently acts as a buffer or protector substance to Ac-globulin and to prothrombin. More significantly, they call attention to a probable rôle of fibrinogen in the conversion phase of the normal clotting process. Although the prothrombin concentration is not altered detectably by the removal of fibrinogen, Ac-globulin activity is. This qualitative difference, therefore, focuses attention on the Ac-globulin-fibrinogen relationship particularly. It is conceivable that Ac-globulin "molecules" are enveloped by, or form a complex with fibrinogen molecules, in which state the former may be considered to be inactive. With the liberation of small amounts of thrombin, or by adding small amounts of thrombin to plasma, the fibrinogen is converted to fibrin, thereby releasing "active" Ac-globulin (serum type) "molecules." Such an hypothesis is consistent with many observed, but hitherto unexplained phenomena in blood clotting, and can serve as a starting point for sound speculation and experiment. Recently, for example, Quick and Stefanini(4) have shown that the effect of rabbit labile factor on stored human plasma is increased over that on fresh plasma. They have called attention to the similarity between this observation and that of Ware, Murphy, and Seegers(5) regarding the conversion of an inactive plasma Ac-globulin to an active serum Ac-globulin. The release of fibrinogen from an Ac-globulin-fibrinogen complex incident to the storage of plasma could account for the increase in the sensitivity of the stored human plasma to labile factor. Similarly, the influence of varying amounts of thrombin on Ac-globulin activity in oxalated bovine plasma(6) could be explained by the removal of fibrinogen. The conversion of an inert pro-enzyme plasma Ac-globulin to an active serum-type Ac-globulin may be mediated by thrombin, by freezing and thawing, or, in all probability, by any chemical or physical agent which effectively removes fibrinogen without concomitant removal of Ac-globulin. Quantitative studies of this problem are in progress. Further, it is tentatively postulated that with additional study of the fibrinogen-Ac-globulin relationship, the differences and incongruities between the various accelerator factors described may, in part at least, be reconciled.

The increase of Ac-globulin activity subsequent to the removal of fibrinogen is of considerable practical importance in the routine assay of plasma Ac-globulin. From the above discussion, it would seem evident that the Ac-globulin activity in plasma is a function of several variables, of which fibrinogen, and agents which alter it, is of appreciable significance. As a corollary, it would seem equally evident that Ac-globulin as a quantity or entity can be measured accurately only in serum or in completely defibrinated plasma.

Summary. Evidence is presented which indicates that fibrinogen serves as a buffer or protector substance to Ac-globulin. The removal of fibrinogen from plasma by the addition of thrombin or by freezing and subsequent thawing enhances Ac-globulin activity. The data provide a probable explanation for the conversion of an inactive pro-enzyme accelerator in plasma to an active accelerator in defibrinated plasma and in serum.

5. Ware, A. G., Murphy, R. C., and Seegers, W. H., *Science,* 1947, v106, 618.

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Distribution of an Unidentified Antithyrotoxic Factor in Materials of Plant and Animal Origin.* (17918)

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Available data indicate that requirements for a number of nutrients are markedly increased in the hyperthyroid animal(1). This is particularly true for some of the B vitamins. An increased requirement for thiamine (2,3), pyridoxine(4), pantothenic acid(4), folic acid(5) and, more recently, vitamin B_{12} has been demonstrated following the administration of large doses of thyroactive substances. In addition to the above, requirements are increased for at least one additional factor as well. This substance which has been termed the "antithyrotoxic factor of liver" will counteract completely the growth retardation of immature rats and mice fed massive doses of thyroid in conjunction with a purified ration containing casein as the dietary protein and sucrose as the dietary carbohydrate(6,7). It is present in considerable concentration in the water-insoluble fraction of liver (liver residue) and is apparently distinct from any of the known nutrients(8-11). In the present communication data are presented on the comparative effects of liver residue and other materials of plant and animal origin on the gain in body weight of immature hyperthyroid rats.

Procedure and results. The basal ration employed in the present experiment consisted of sucrose 73.0%; casein,† 22.0%; salt mixture,‡ 4.5%; and U.S.P. desiccated thyroid,§ 0.5%. To each kg of the above were added the following synthetic vitamins: thiamine hydrochloride, 72 mg; riboflavin, 9 mg; pyridoxine hydrochloride, 15 mg; calcium pantothenate, 67.2 mg; nicotinic acid, 60 mg; 2-methyl-naphthoquinone, 5 mg and choline chloride, 1.2 g‖. Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson), 500 mg; alpha-tocopherol acetate, 1.5 mg and a vitamin A-D concentrate containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D¶. The various supplements to be tested were incorporated in the basal ration as per cent of the total diet, replacing an equal amount of sucrose. The supplements tested and the levels at which they were fed are listed in Table I. Male and female rats of the Long-Evans strain were selected at 22 to 25 days of age for the present study. Animals were kept in metal cages with raised screen bottoms to prevent access to feces, and were fed the various rations *ad lib.* Feeding was

* Communication No. 262 from the Department of Biochemistry and Nutrition, University of Southern California.

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10. Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 209.
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† Vitamin Test Casein, General Biochemicals, Inc.

‡ Hubbel, Mendel, and Wakeman Salt Mixture, General Biochemicals, Inc.

§ Thyroid Powder, U.S.P., Armour and Co.

‖ In view of the increased requirements for thiamine, pyridoxine, and pantothenic acid in the hyperthyroid rat(4), these vitamins were administered in excessive amounts in the present experiment in order to assure an adequacy of these factors in the diet.

¶ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vit. A and 80,000 U.S.P. units of vit. D per g.

TABLE I.

Effects of Liver Residue and Other Materials of Plant and Animal Origin on the Gain in Body Weight of Immature Hyperthyroid Rats. Eight animals were placed in each experimental group, 10 in control group on basal ration without thyroid, and 12 in control group on basal ration with thyroid.

Supplements fed with basal ration*	Initial body wt, g	Avg gain in body wt in 28 days, g†
♂		
(Basal ration without thyroid)	45.9	105.3 ± 5.2 (10)
0	47.1	51.0 ± 3.4 (9)
Vit. B_{12}	46.4	53.4 ± 3.6 (6)
Synthetic vit. mixture‡	46.3	81.5 ± 2.4 (5)
Heart	46.1	60.7 ± 2.1 (5)
Whole egg	46.4	63.3 ± 3.4 (5)
Muscle (beef flank)	46.3	63.5 ± 3.7 (6)
Thymus	46.0	67.0 ± 3.5 (6)
Streptomycin mash	46.2	67.3 ± 3.0 (7)
Placenta	46.5	72.3 ± 5.8 (6)
Brain	46.4	73.8 ± 4.4 (7)
Duodenum	46.3	75.5 ± 4.5 (6)
Sardine meal	46.3	76.9 ± 2.1 (8)
Soybean flour§	46.1	91.4 ± 3.4 (8)
Kidney	45.9	110.2 ± 4.1 (7)
Liver residue‖	45.8	112.9 ± 4.5 (7)
Aureomycin mash	46.0	118.3 ± 4.9 (8)
♀		
(Basal ration without thyroid)	44.0	91.9 ± 3.6 (10)
0	47.8	44.3 ± 3.6 (10)
Vit. B_{12}	46.2	46.0 ± 3.1 (6)
Synthetic vit. mixture‡	46.9	75.7 ± 1.9 (5)
Aureomycin HCl (500 mg per kg/diet)	46.4	46.7 ± 3.2 (4)
Aureomycin HCl (100 mg per kg/diet)	46.4	49.3 ± 3.3 (5)
Whey	45.9	52.9 ± 3.6 (5)
Alfalfa	46.2	60.6 ± 3.7 (8)
Yeast	46.5	62.4 ± 3.0 (8)
Penicillin mash	47.0	74.6 ± 3.2 (8)
Soybean flour§	46.4	84.3 ± 4.0 (5)
Liver residue‖	45.9	95.4 ± 3.9 (7)
Aureomycin mash	45.8	103.0 ± 4.1 (8)

The values in parentheses indicate the number of animals which survived and on which averages are based.

* The indicated factor constituted 10% of the diet except in the case of vit. B_{12} fed at a level of 30 γ per kg of diet, and aureomycin HCl fed at levels of 100 and 500 mg per kg of diet. Supplements were obtained from the following sources: Cobione (Crystalline Vit. B_{12} Merck); extracted liver residue, Wilson Laboratories; aureomycin mash, APF-5, Lederle Laboratories; kidney, Viobin Corporation; heart substance powder, Armour and Co.; powdered whole egg, Washington Cooperative Farmers Assn.; beef flank, Southern Calif. Gland Co.; thymus powder, Armour and Co.; streptomycin mash, Bi-Con APF 3+, Chas. Pfizer and Co.; placenta powder, Armour and Co.; brain powder, Armour and Co.; duodenum, Viobin Corporation; sardine meal, Van Camp Corporation; soya powder, El Molino Mills; dry cheese whey solids, Western Condensing Co.; alfalfa concentrate, Test Laboratories; brewers' type yeast No. 200, Anheuser-Busch; and penicillin mash, dried penicillin mycelia, Eli Lilly and Co. We are indebted to Dr. E. L. R. Stokstad of Lederle Laboratories for the crystalline aureomycin HCl employed in the present experiment.

† Including stand. error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

‡ The following vitamin supplements were added per kg of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; biotin, 4 mg; folic acid, 10 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg; 2-methyl-naphthoquinone, 10 mg; and vit. B_{12} (Cobione), 30 γ.

§ Full fat soybean flour containing approximately 22% fat.

‖ Extracted liver residue consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

continued for 28 days or until death, whichever occurred sooner. Results are summarized in Table I.

In agreement with earlier findings massive doses of thyroid retarded significantly the gain in body weight of immature rats fed a purified ration containing casein as the dietary protein and sucrose as the dietary carbohydrate. No increase in growth resulted from the administration of crystalline vitamin B_{12}; supplements of all the known B vitamins, however, promoted a significant increase in body weight although the growth increment was less than that obtained with rats fed the basal ration with thyroid omitted. Liver residue, desiccated and defatted kidney and a fermentation product derived from cultures of *Streptomyces aureofaciens* (APF-5)** completely counteracted the growth retardation of immature rats fed massive doses of thyroid under conditions of the present experiment. Some increase in body weight over that obtained on the basal ration was observed following the administration of desiccated and defatted heart, beef flank, thymus, placenta, duodenum and brain as well as powdered whole egg, fish meal, whey, alfalfa, yeast, streptomycin residue (Bi-Con APF-3+) and a sample of penicillin mash. Gain in body weight on these supplements, however, was significantly less than that obtained on the basal ration with thyroid omitted. Soybean flour as previously reported(12) showed considerable activity as a source of growth-promoting factor for the hyperthyroid rat. Crystalline aureomycin HCl at levels of 100 or 500 mg per kg of diet, however, was without significant effect.

Discussion. Previous findings indicate that whole liver contains one or more factors apparently distinct from any of the known nutrients whose requirement is increased in hyperthyroid rats fed a purified ration containing casein as the dietary protein and sucrose as the dietary carbohydrate. A deficiency of this factor results in a marked retardation of growth in the immature thyroid-fed rat which may be completely counteracted

** The material employed, which was kindly provided by Dr. E. L. R. Stokstad, contained approximately 5 mg of aureomycin per g. Tests were subsequently conducted to determine the growth-promoting effects of smaller doses of this material. APF-5 when fed at a level of 2% of the diet had no significant effect on rate of growth. APF-5 at a 5% level markedly increased the gain in body weight but the weight increment was less than that obtained with the 10% supplement.

12. Ershoff, B. H., *J. Nutrition*, 1949, v39, 259.

by the administration of desiccated whole liver or its water-insoluble fraction but not by supplements of the various known nutrients (8-11). Results of the present experiment indicate that desiccated and defatted kidney and a fermentation product derived from cultures of *Streptomyces aureofaciens* (APF - 5) were as active as liver residue in counteracting the growth retardation of immature rats fed massive doses of desiccated thyroid. Full fat soybean flour also showed considerable activity in this regard. Stokstad *et al.* (13) have recently observed that aureomycin mash contains a factor apparently distinct from any of the known nutrients which is essential for the optimal growth of chicks. The activity of aureomycin mash under conditions of the present experiment suggests the possibility that the "antithyrotoxic factor of liver" and the chick growth factor of Stokstad *et al.* may be one and the same factor. Crystalline aureomycin HCl, however, which exerts growth-promoting activity in the chick(14), turkey and pig(15) is inactive as a source of growth-promoting factor for the hyperthyroid rat. The ineffectiveness of whey (Table I) appears to distinguish the active principle in the present experiment from the "whey factor" described by Hill(16). Some increase in body weight over that obtained on the basal ration was observed in thyroid-fed rats under conditions of the present experiment following the administration of desiccated and defatted heart, beef flank, thymus, placenta, duodenum or brain as well as powdered whole egg, fish meal, whey, alfalfa, yeast or streptomycin and penicillin mash. The gain in body weight of rats fed these supplements, however, was less than that of animals receiving an additional supply of the known B vitamins suggesting that the growth-promoting effect of these supplements may have been due, at least in part, to their content of known B vitamins.

Summary. Growth was markedly reduced in hyperthyroid rats fed a purified ration containing casein as the dietary protein and sucrose as the dietary carbohydrate. The retardation in growth was completely counteracted by the administration of a water-insoluble fraction of liver, desiccated and defatted kidney and a fermentation product derived from cultures of *Streptomyces aureofaciens*. Full fat soybean flour also showed considerable activity. Crystalline aureomycin HCl was inactive. Other materials of plant and animal origin showed little if any activity. The protective factor was apparently distinct from any of the known nutrients.

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Absorption, Distribution and Excretion of Phenindamine (Thephorin). (17919)

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The compound, phenindamine (2-methyl-9-phenyl 2,3,4,9 tetrahydro-1-pyridindene), is available under the trade name Thephorin for the treatment of various allergic conditions. Its antihistaminic and other pharmacologic properties have been described by

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Lehmann(1). However, despite its wide therapeutic usage, little is known in regard to its absorption, distribution and excretion. The results of such studies on rats and its urinary excretion in humans are presented in this report.

Procedure and results. Estimation of Phenindamine: The compound was determined quantitatively by the Brodie and Udenfriend methyl orange technic for basic amines (2) using technical benzene as a solvent. It was found necessary to modify the method for estimating phenindamine recovered from biologic samples because it became apparent in our preliminary studies that considerable material extracted by the benzene reacted as phenindamine in the analytical procedure. Previous experience with the estimation of other basic amines (Demerol(3), Methadone (4), and Pyribenzamine(5)) by the methyl orange technic suggested that interfering substances could be removed by simply washing the solvent extract with a buffer at an appropriate pH. With phenindamine, this was accomplished by washing the benzene extract of tissue 4 times with an acetate buffer at pH 5.5. Recovery of known amounts of phenindamine added to various tissue homogenates averaged 85 $\pm$ 10%. Tissue blanks averaged from 0.005 optical density for muscle to 0.025 for brain.‡

Procedure for Tissues. Weighed samples of the tissue to be studied were diluted 1 to 15 with M/50 sodium fluoride solution and thoroughly minced in a Potter homogenizer. One cc of N/1 NaOH and 10 cc of benzene were added to 5 cc of tissue homogenate in a glass stoppered centrifuge tube and shaken for 3-5 minutes. The mixture was centrifuged and the aqueous layer removed by aspiration. Five cc of M/2 acetate buffer pH 5.5 were added to the benzene layer. After shaking thoroughly, the buffer phase was removed by centrifugation and aspiration. The benzene layer was washed three additional times in the same manner. A 7 cc aliquot was transferred to a glass stoppered tube containing 0.5 cc of the methyl orange reagent. Isoamyl alcohol (0.25 cc) was added and the tube shaken for 1 minute. The tube was then centrifuged and the methyl orange layer completely removed by aspiration. The benzene layer was transferred to another tube and a 5 cc aliquot of this solution was added to a colorimeter tube containing 1 cc of 2% H_2SO_4 in absolute alcohol. The intensity of the color thus developed was read in a Coleman Junior Spectrophotometer at 540 mu and the amount of phenindamine present was estimated by reference to a standard curve.

Specificity of Method. Evaluation of the specificity of the method was carried out according to Brodie(2). An unknown compound may be identified with a known substance by comparing their distribution ratios between an organic solvent and a series of aqueous buffers of varying pH. Similar ratios would indicate that the two substances are alike since the probability is rather remote for 2 different compounds to have identical solubility properties. Accordingly, this principle was applied to the phenindamine recovered from various organs of several rats which were given the compound by the intraperitoneal route. After extraction of the minced tissue with benzene, an aliquot of the benzene layer was analyzed for methyl orange reactants. Aliquots of the same solution were equilibrated with a series of phosphate buffers and the amount of methyl orange reactant remaining in the benzene was determined. The ratios of the amount of methyl orange reactant remaining in benzene before and after equilibration with the buffers were compared with those obtained with a known sample of phenindamine.

From the results in Table I, it became obvious that the alkaline benzene extract of

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2. Brodie, B. B., and Udenfriend, S., *J. Biol. Chem.*, 1945, v158, 705.

3. Leong Way, E., Gimble, A. I., McKelway, W. P., Ross, H. and Ellsworth, H., *J. Pharmacol.*, 1949, v96, 477.

4. Leong Way, E., Sung, C. Y., and McKelway, W. P., *J. Pharmacol.*, 1949, v97, 222.

5. Leong Way, E., and Dailey, R. E., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 423.

‡ Benzene on standing may yield higher brain blanks and must be checked at frequent intervals for such changes.

TABLE I.

Distribution Ratios of Phenindamine Remaining in Benzene to Total Drug (Added or Recovered) After Equilibration with Various Buffers.

Sample description	No. of buffer washes	0.5 M phosphate, pH 8	7	6	5
Added to water	0	1.01	1.04	.99	.98
Recovered from liver	0	1.01	.99	.77	.59
" " kidneys	0	1.03	1.03	.87	.72
" " lungs	0	.89	.88	.79	.53
" " "	1	—	.99	.88	.59

Sample description	No. of buffer washes	0.5 M acetate, pH 5.5	5.1	4.1	3.3
Added to water	2	.98	.97	.72	.36
Recovered from liver	3	.85	.80	.51	.29
" " "	4	—	.99	.71	.37
" " kidneys	3	.99	.91	.66	.32
" " lungs	3	.86	.84	.61	.37
" " "	4	—	.97	.68	.40
" " spleen	3	.98	.91	.73	.35

tissues contained material which behaved differently from phenindamine, since the distribution ratios of the methyl orange reactant recovered from animals differed considerably from those of a known sample of phenindamine. In all probability this was due to the presence of degradation products which reacted as the parent compound. It was subsequently found that the interfering substances could be removed by washing the benzene extract of tissues 3-4 times with M/2 acetate buffer, pH 5.5. After such treatment the solubility properties of the methyl orange reactant remaining in the benzene extract of tissues were found to resemble those of a known sample of phenindamine (Table I).

Absorption of phenindamine. The rate of oral absorption of phenindamine was studied by measuring its rate of disappearance from the gastrointestinal tract of rats fasted for 18-24 hours. A dose of 100 mg/kg was administered by stomach tube. The animals were sacrificed at various time intervals and their gastrointestinal tracts were removed, diluted to 500 cc and thoroughly minced in a Waring Blendor. The amount of drug still present in a sample of the homogenate was determined as previously described. It was assumed that destruction of the drug in the gut did not occur, since incubation studies of phenindamine (added or recovered) with the enteric tract plus its contents did not reveal any alteration of the original phenindamine concentration. The results as shown in Fig. 1 indicate that approximately 75% of a phenindamine dosage disappears from the gastrointestinal tract within 4 hours. Since approximately 10% of the dosage was still present in the gastrointestinal tract after 24 hours, excretion of the compound by the gut must be considered. In contrast, our previous results with another antihistaminic, Pyribenzamine, revealed that practically all of the compound had disappeared from the enteric tract 4 hours after oral administration (5).

Tissue distribution of phenindamine. The distribution of phenindamine was determined on rats. Following the administration of

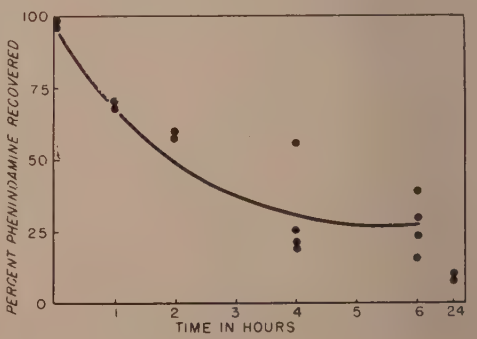

FIG. 1.

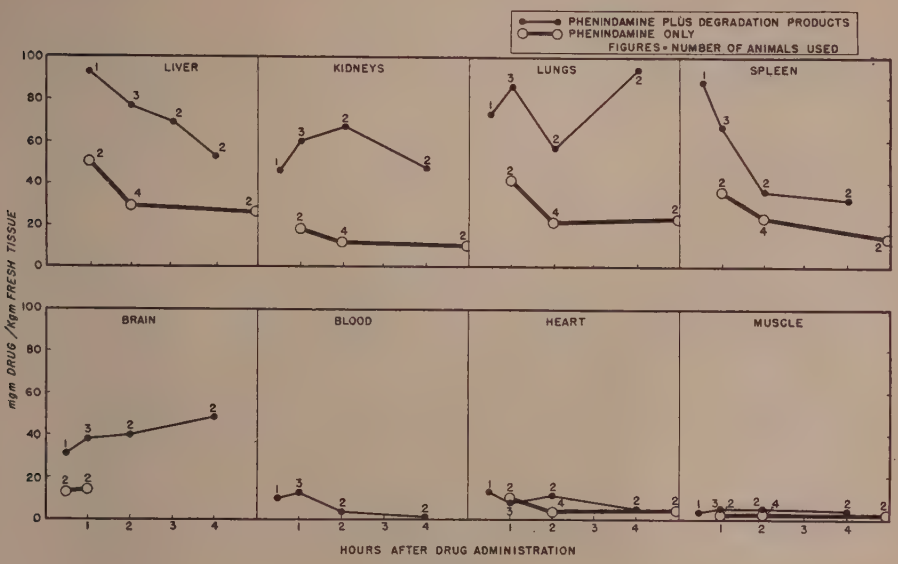

FIG. 2.

phenindamine 45 mg/kg intraperitoneally, the animals were sacrificed at definite time intervals. Various organs were removed and analyzed for phenindamine content in the manner previously described. A series of tissue levels were also determined omitting the quadruple buffer wash of the benzene extract in the procedure. The tissue levels of phenindamine obtained with and without the buffer wash are summarized in Fig. 2. The two methods differ considerably as to the absolute amount of phenindamine present in each tissue, but show a similar trend as to the ability of various organs to concentrate the drug. Since the unwashed benzene extract measures the compound plus its degradation products which also react with methyl orange, whereas the washed benzene extract essentially measures only phenindamine, it is quite apparent that phenindamine is very rapidly metabolized in the body. The compound appears to leave the blood rapidly and localize in tissues. High concentrations of the phenindamine were found in the lungs, liver, kidneys and spleen. Appreciable concentrations were also present in brain, whereas the levels in the blood, heart and muscle were barely detectable. Appreciable levels of phenindamine were still demonstrable in the lungs even after 5 hours. These findings appear to support the findings of Lehmann on guinea pigs, that phenindamine has a longer duration of action than most antihistamines(1). However, extensive storage of the compound does not seem to occur. These conclusions are based on our experimental findings made on 4 rats which received 20 mg/kg of phenindamine twice daily for 4 consecutive days. On analyzing the tissues of the animals for phenindamine 24 hours after the last administration, no phenindamine could be detected. These results with phenindamine resemble our previous results with Pyribenzamine(5), although the latter appears to disappear more rapidly from the body.

Excretion of phenindamine. The kidney appears to play only a minor role in the excretion of phenindamine. In total 24 hour urine samples collected from 3 humans (males) following administration of 50 mg orally, less than 2% of the total dosage was recovered as phenindamine. Likewise, in 2 rats, less than 2% of the total dose was re-

covered in the urine 24 hours after giving 100 mg/kg, intragastrically. Since less than 10% of the dose was recovered concomitantly from the gastrointestinal tract and its contents, it is concluded that for the most part phenindamine is completely metabolized in the body.

Summary. Phenindamine (Thephorin) was determined in biologic material with a high degree of specificity by a modification of the Brodie methyl orange technic. Negligible amounts of phenindamine can be recovered in the urine of humans following oral administration. In studies on rats the compound appears to be rapidly absorbed from the gastrointestinal tract. The compound rapidly leaves the blood and concentrates in tissues. High concentrations of the drug were found in the lungs, liver, kidneys and brain. Extensive accumulation of phenindamine did not occur after repeated administration of the compound twice daily for 4 consecutive days. It is concluded that the compound is metabolized to a considerable extent in the body since less than 12 per cent of the total dose of phenindamine could be recovered unchanged in the urine and feces 24 hours following oral administration.

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Relationships of Vital Capacity and Ventilatory Measurements to Physical Fitness in Patients with Cardio-Respiratory Diseases.* (17920)

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Previous studies have shown a direct relationship between physical fitness scores of working capacity and the availability of oxygen to the tissues in an heterogeneous group of ambulatory patients with cardio-respiratory diseases(1). The clinical evaluation of disability in these patients varied from virtually none to complete and total disability, with the majority comprising the intermediate ranges. Physical fitness was scored from an integration of the most reproducible characteristics of circulatory and pulmonary performance during standardized exercise. The first of these was the duration of time that moderate exercise on a treadmill ergometer could be tolerated (up to an arbitrary maximum of 10 min.). The other criteria were the mean respiratory efficiency (volumes % of oxygen absorbed per unit volume of air ventilated) during exercise, and the total number of heart beats for the first 3 minutes of recovery(2). Others have found significant relationships of the vital capacity, the maximum breathing capacity and the breathing reserve to the awareness of dyspnea in patients with pulmonary diseases(3, I-III). In view of these considerations, it becomes important to ascertain how well vital capacity, and various measures of ventilatory performance commonly used in evaluating

* Aided by grant from National Institute of Health, Contract H-222.

† Bertha Hochstetter Buswell Research Fellow in Medicine.

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TABLE I.
Relations of Ventilatory Measurements to Physical Fitness.
(Simultaneous values in 44 patients).

Factors	Mean ± St. dev.	Range, min.–max.	Coef. of variation, %	Coef. correlation with physical fitness index, r ± st. error*	p, %
Physical fitness index†	11.6 ± 5.4	1.3– 21.5	46.6		
Vital capacity, Obs. × 100 / Pred.	73.3 ± 19.3	21 –105	26.3	+.587 ± .099	<.0001
Max. mask breathing capacity, L/m²/m	24.7 ± 7.4	8.3– 39.2	30.0	+.395 ± .128	<.27
Mean exercise ventilation, L/m²/m	9.46 ± 2.08	4.2– 13.4	21.9	—.021 ± .145	
Breathing requirement, %	41.4 ± 14.8	19 – 79	35.7	—.477 ± .117	<.006
" reserve, %†	58.8 ± 14.6	21 – 81	24.8	+.458 ± .120	<.014
Mean exercise mid-capacity, pCO_2, mm Hg	33.7 ± 7.5	20 – 52	22.2	+.410 ± .126	<.13
Ventilation, % of total	73.2 ± 9.5	54 – 88	12.9	+.181 ± .147	<23

* Standard error of coefficient of correlation equals $\frac{1-r^2}{\sqrt{N-1}}$

† The mean normal values of the physical fitness index and breathing reserve at 1.73 mph. level walking are 18.9 ± 2.8 and 71.6 ± 6.4, and at 2.6 mph. level walking, 19.4 ± 3.8 and 74.8 ± 4.6, respectively.

pulmonary function, correlate with the physical fitness index in patients selected at random.

Methods. For this purpose the exercise data on 44 patients with a variety of cardiorespiratory diseases have been analyzed. They ranged in age from 15 to 61 years. The chief clinical categories of disease represented were: congenital cardiovascular disease, 8; acquired heart diseases, 12; pulmonary diseases, 16; and miscellaneous diseases affecting cardiorespiratory functions, 8. Vital capacity was measured in the usual manner, and expressed as per cent of the predicted values for age, sex, and height(3, I). The maximum mask breathing capacity was obtained at the end of the treadmill test in order that the breathing requirement‡ and reserve values would be based upon the same equipment of face mask and valves as were used for the ventilation measurements during exercise. (Since high velocity valves were not employed, the values were lower than those generally reported in the literature, and the mean normal value for adults was 29.8 ± 5.6 liters per square meter of body surface area per minute, STPD). The exercise tests (*level* walking) were the same as described previously(4). Since the degree of disability varied from almost none to complete, 24 patients were able to walk for 10 minutes at 2.6 m.p.h., 9 were able to walk for 10 minutes at 1.73 m.p.h., and the remainder were unable to complete the prescribed period of 10 minutes at either speed tested. The mid-capacity (intra-pulmonary) ventilation percentage was derived from:

$$\frac{\text{Insp. } O_2\% - \text{Exp. } O_2\% \times 100}{\text{Insp. } O_2\% - \text{Mid-capacity } O_2\%}$$

3. (I) Baldwin, Cournand, Richards, D. W., Jr., *Medicine*, 1948, v27, 243; (II) Baldwin, Cournand, Richards, D. W., Jr., *Medicine*, 1949, v28, 1; (III) Baldwin, Cournand, Richards, D. W., Jr., *Medicine*, 1949, v28, 201.

‡ The breathing requirement equals:

$$\frac{\text{average exercise minute ventilation volume}}{\text{maximum mask breathing capacity}} \times 100$$

whereas the breathing reserve equals 100 minus the breathing requirement. Gas volumes are corrected to square meters of body surface area, STPD, to aid estimation of oxygen consumption(4).

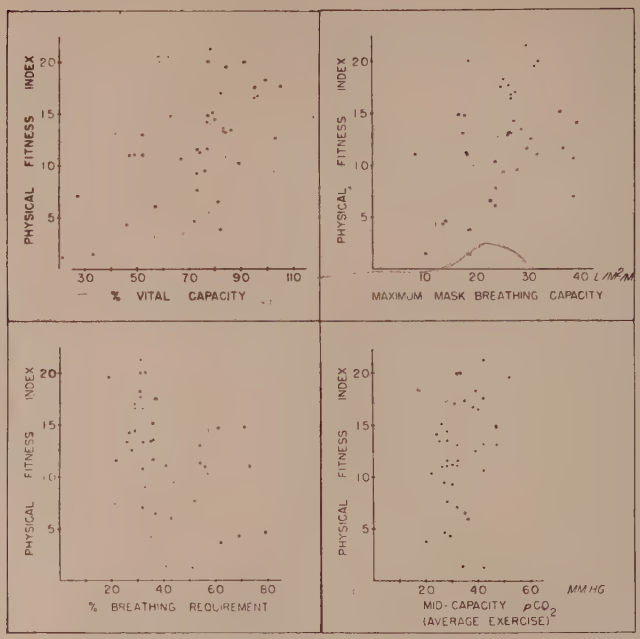

FIG. 1.

Relationships of the physical fitness index to the percentage vital capacity, maximum mask breathing capacity, percentage breathing requirement and mid-capacity (mixed alveolar) pCO_2 during exercise.

Hence, the percentage dead space ventilation equalled 100 minus the percentage mid-capacity ventilation. Statistical correlations were made by the product-moment method, and scatter graphs of significant relationships are included. The awareness of dyspnea, and the relative intensity of distress were evaluated in each patient.

Results. The statistical analyses of the results obtained are presented in Table I. The least variable factor was the percentage mid-capacity ventilation during exercise. This represented the intra-pulmonary portion of the total ventilation, whereas the remainder represented the ventilation of the physiological dead space. The most variable factor was the physical fitness index. A significant relationship was found between the physical fitness index and the vital capacity expressed as the percentage ratio of the observed to predicted volumes (Fig. 1). Relationships of lesser significance were also demonstrated between the physical fitness index and the maximum mask breathing capacity, breathing requirement and breathing reserve, as well as the mean exercise mid-capacity (mixed alveolar) pCO_2. Neither the average minute ventilation, nor the mid-capacity portion of the total ventilation, showed any relationship to the fitness index.

Whereas only 30% of the patients who were able to walk for 10 minutes experienced dyspnea, all patients who were unable to walk for 10 minutes complained of dyspnea. In general, the more intense the dyspnea, the more marked was the observed reduction in exercise tolerance.

Comments. Of the several ventilatory measurements appraised in this heterogeneous group of patients, more or less disabled by cardio-respiratory diseases, the percentage vital capacity showed the best correlation

4. Bruce, R. A., Lovejoy, F. W., Jr., Pearson, R., Yu, P. N. G., Brothers, G. B., and Velasquez, T., *Clin. Invest.*, 1949, v28, 1423.

with the physical fitness index of exercise performance. The vital capacity has been expressed in these terms in order to correct for variations in individuals due to age, sex, and height. This relationship to the ability to perform moderate exercise of level walking is not a perfect one, however. Some patients with either acquired or congenital heart disease may have appreciable venous admixture via abnormal channels, or perfusion of inadequately ventilated alveoli, which permits incomplete saturation of the arterial blood with oxygen. Thus these individuals may have diminished exercise tolerance from hypoxemia independent of significant changes in vital capacity. Furthermore, the vital capacity volume may be a poor index of exercise performance in some emphysematous patients who exhibit a nearly normal vital capacity volume, but are unable to expel the air at a normal velocity because of diminished pulmonary elasticity. In such individuals the maximum breathing capacity is reduced and affords a more reliable appraisal of ventilatory performance. In this heterogeneous group of randomly selected patients, it should be noted that the breathing requirement showed a correlation with the fitness index which the mean exercise ventilation volume failed to exhibit.

Although in a previous study the physical fitness index was found to be proportional to the physically dissolved oxygen in the blood(1), we have not been able to demonstrate a direct relationship between the vital capacity and the arterial pO_2. Nevertheless, the simplified measure of working capacity used herein may reveal relationships to some of the physiological factors, which in an integrated fashion, determine the overall capacity for exertion. The interrelations of such factors, however, do not always follow because of the multiplicity of pathological processes which are responsible for functional impairment.

Summary. Simultaneous analyses of various ventilatory measurements in 44 randomly selected patients with cardio-respiratory diseases have been assembled. A significant relationship was found between the physical fitness index and the percentage ratio of the observed to predicted vital capacity. The limitations of this relationship, as well as other relationships of physical fitness score to various ventilatory measurements, are discussed.

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Cardiovascular Changes Induced by Rapid Expansion of the Extracellular Fluid. (17921)

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(Introduced by Homer W. Smith)

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It has been shown(1) that marked alterations in glomerular filtration rate and renal blood flow follow the expansion of extracellular fluid which occurs when large volumes of a modified Locke's solution are infused intravenously in dogs. In order to assess the role of general cardiovascular changes in this response we have examined vascular pressures and volume during and after such infusions.

Methods. Two series of observations were made on each of 4 trained, unanesthetized female dogs weighing 12 to 15 kg. In 5 experiments 1100 cc, and in 3 experiments 1600 cc, of a modified Locke's solution* were

1. Wesson, L. G., Anslow, W. P., Jr., Raisz, L. G., Bolomey, A. A., and Ladd, M., *Am. J. Physiol.*, in press.

* The solution contains 153 mEq/l of sodium, 134 mEq/l of chloride, 27 mEq/l of bicarbonate, 4 mEq/l of potassium, 4 mEq/l of calcium, and 100 mg/100 cc of glucose.

infused at rates varying between 140 and 220 cc/min. Femoral arterial pressure was measured by a mercury manometer. Venous pressure was measured by the method of Birch and Sodeman(2) with a plastic catheter of small diameter inserted in the jugular vein except in one experiment where the saphenous route was employed. The catheter was of such a length that the tip lay in the great veins or right auricle. The reference point for venous pressure was taken as a point mid-way between the sternal angle and the surface of the back between the spines of the scapula. Plasma protein concentration was determined by the copper sulfate specific gravity method(3).

Results. Venous pressure rose from control values of 3.5 to 8.0 cm of water to peak values of 13 to 38 cm, 3.5 to 8 minutes after the infusion was begun and always before it had been completed. When 1600 cc were given the peak value was higher than when 1100 cc were infused.† Regardless of the height attained, venous pressure decreased rapidly after the peak and attained stable values within 13 to 25 minutes after completing the infusion. In 6 experiments the pressure decreased to or below the control level. In 2 experiments pressure stabilized at values 4.5 and 5 cm above control values, the absolute levels remaining below 8 cm of water.

Mean arterial pressure increased from control values of 90 to 120 mm of mercury to 115 to 155 mm, the average increase being 30 mm. The pressure decreased from this peak to within 10 mm above or below the control within 30 minutes in all but 2 experiments. In one of these the pressure showed a progressive increase from 85 to 130 mm in 2 hours. In the second the pressure remained elevated at a level intermediate between the peak and the control values.

The plasma protein concentration reached its minimal value of 46 to 61% of the control immediately after completing the infusion (no values were recorded during the infusion). This circumstance corresponds to an expansion of plasma volume by 64 to 117%. The hematocrit at this time had decreased to 51 to 73% of the control value. The disproportion between the changes in protein concentration and hematocrit reflects the fact that isotonic electrolyte solutions increase the plasma volume without changing red cell volume. After 70 minutes the plasma protein concentration had returned to 82 to 87% of the control (corresponding to plasma volume expansion of 15 to 20%), and hematocrit to 90 to 107% of the control. No changes were observed in the next 50 minutes, after which time observations were discontinued.

The heart rate usually increased from control levels of less than 100 to 130-150 beats per minute during the infusion and decreased rapidly thereafter. The animals appeared to tolerate the infusion well. There was no evidence of respiratory or circulatory distress. The changes in urine flow were similar to those described elsewhere(1).

Discussion. Infusion of isotonic solutions in the dog at a rate of 200 cc/min is rapidly accommodated by the circulation. Landis, Brown, Fauteux and Wise(4) inferred that saline could escape from the vascular compartment at rates of 4.5 to 6.0 cc/kg per min., since an infusion at this rate was necessary to maintain a constant elevation of venous pressure. Estimating blood volumes as 8% of body weight in the experiments reported here it can be calculated from changes in the plasma protein concentration that fluid escaped from the vascular compartment at rates

2. Birch, G. E., and Sodeman, W. A., *J. Clin. Invest.*, 1937, v16, 845.

3. Phillips, R. A., Van Slyke, D. D., Hamilton, P. B., Dole, V. P., Emerson, K., Jr., and Archibald, R. M., *J. Biol. Chem.*, 1950, v183, 305.

† The lowest peak occurred in the one experiment where venous pressure was measured from the saphenous vein. This circumstance raises the question as to whether the peak pressures measured from the jugular vein in these experiments represent generalized systemic changes. Since the infusion was given under pressure through the contralateral jugular vein it is possible that local reflections of the infusion pressure at the tip of the catheter from which venous pressure was recorded were responsible for the high peaks obtained via the jugular route.

4. Landis, E. M., Brown, E., Fauteux, M., and Wise, C., *J. Clin. Invest.*, 1946, v25, 237.

as high as 55 cc per minute in a 13 kg dog. This value is comparable to that reported by Landis and his co-workers.

The increases in filtration rate and renal plasma flow which follow such infusions as we have studied do not attain their maximal values until an hour or more after the termination of the infusion(1). Slow infusions do not increase the venous or arterial pressure, and the increments induced by rapid infusions have generally disappeared within 30 minutes after the end of the infusion. We conclude, therefore, that except for the early abrupt increase in filtration rate, which may in part be related to increased arterial pressure and hemodilution, the changes induced in renal function by such infusions are not related to changes in venous or arterial pressure, increased plasma or blood volume or protein dilution.

Summary. The intravenous infusion of 1100 to 1600 cc of a modified Locke's solution at rates of 140 to 220 cc/min. in dogs causes only a transient increase in venous and arterial pressure and pulse rate. These values usually return to control levels within half an hour. Plasma and blood volume as measured by changes in plasma protein concentration and hematocrit are increased markedly at first, but return to within 20% of control values 70 minutes after the infusion.

These systemic changes do not coincide in time with the increase in filtration rate and renal plasma flow induced by such infusions, and it is concluded that the changes in renal function cannot be attributed to them.

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On the Mechanism of Action of Aminopterin.* (17922)

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In studies on the metabolism of folic acid and its derivatives, it was observed that rat liver slices form from pteroylglutamic acid (PGA) a substance or substances utilized for growth by *Leuconostoc citrovorum* 8081(1). The existence of a factor of unknown chemical nature, referred to as citrovorum factor (CF), was first described by Sauberlich and Baumann(2) and its relation to folic acid was indicated by Sauberlich(3). Using the liver slice system, conditions influencing the synthesis of CF were studied(1), and it was found that the presence of certain reducing agents, particularly ascorbic acid, stimulated the formation of CF, both from synthetic PGA and from compounds present in normal rat liver. It was suggested that a reductive step may be involved in the enzymatic synthesis of CF. The 4-amino analogue of PGA, aminopterin, is one of the most potent inhibitors of the functions of PGA. However, in contrast to the reversible antagonism that often exists between metabolites and structural analogues that interfere with their biological action, aminopterin proved to be reversed very inefficiently by PGA. Thus, Franklin, *et al.*(4) reported that even high levels of PGA were unable to counteract the toxic effects in mice caused by feeding aminopterin at a level of 1 mg per kg of diet.

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

1. Nichol, C. A., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 52.

2. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1948, v176, 165.

3. Sauberlich, H. E., *J. Biol. Chem.*, 1949, v181, 467.

4. Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 398.

TABLE I.
Aminopterin Inhibition of Conversion of PGA to Citrovorum Factor in Liver Slices; Comparison of Normal and PGA-deficient Rats.

		Additions to vessels containing liver slices* Citrovorum factor activity—units/gram of tissue					
					Ascorbic acid—10 mg		
	Aminopterin, μg/vessel		10 μg PGA	100 μg PGA		10 μg PGA	100 μg PGA
PGA-deficient rat liver (6)	0	70	1530	2700	180	8450	14000
	6.25		350	720		1190	4000
	12.5		200	880		880	3350
	25			430			1920
	50	50		200			1190
	50†			2320†			17200†
Normal rat liver (4)	0	1310	1770	2310	3990	5770	7250
	12.5		1200	1410		3280	3330
	25		1110	1690		5150	3480
	50	1240		1160			3510
	50†			2540†			6880†

* The contents of the vessels and their manner of treatment are described in the text. Each vessel contained about 1 g of liver slices from the number of animals indicated by the figure within the parentheses.

† These values represent the levels of CF found when the aminopterin was added at the *end* of the period of incubation. In comparison with the control values, they indicate that aminopterin had no appreciable effect on the response of the microorganism to the CF present.

Also, Higgins(5) observed that daily injection of 50 μg of aminopterin in rats caused signs of acute PGA-deficiency and death in spite of the simultaneous oral administration of PGA in doses as high as 30 mg per day. On the other hand, in the microorganism *Leuconostoc citrovorum,* Sauberlich(6) noted that inhibition of growth by aminopterin was counteracted effectively by concentrates of CF derived from liver or from urine. The possibility that aminopterin inhibits the functions of PGA by interfering with either the conversion of PGA to CF or the utilization of CF, or both, has been the subject of the present investigation.

Methods and results. Experiments in vitro. Weanling albino rats were depleted of PGA by feeding a purified casein-cerelose diet containing succinylsulfathiazole, as described previously(1). The normal rats were raised on a complete stock diet. Liver slice experiments were carried out in small beakers containing 4.5 cc of Krebs-Ringer phosphate solution (pH 7.4) to which appropriate additions of PGA, aminopterin, and ascorbic acid were made; the final volume of fluid was 5 cc in each case. Consecutive slices of liver were placed in the beakers in regular sequence in order to minimize variations in composition; the vessels were weighed before and after the addition of the slices. In all experiments, the incubations were carried out with shaking in a covered water bath at 37° for 2 hours. The ascorbic acid solution was freshly neutralized and was added quickly to the vessels as soon as they had reached the temperature of the water bath. After incubation, the vessels were steamed at 100° for 5 minutes, and the contents of each vessel were ground in a glass homogenizer. The mixture was adjusted to pH 6.5, transferred to a glass-stoppered test tube, diluted to 25 cc, steamed at 100° for 10 minutes, thoroughly mixed and filtered. The clear filtrates were assayed microbiologically with *Leuconostoc citrovorum* 8081, using the basal medium described by Sauberlich and Baumann(2). Turbidimetric readings were made after about 20 hours at 37° using the Klett-Summerson photoelectric colorimeter with a 660 mμ filter.† In all experiments conducted *in vitro* a comparison

5. Higgins, G. M., *Blood,* 1949, v4, 1142.

6. Sauberlich, H. E., *Arch. Biochem.*, 1949, v24, 224.

was made of the production of CF in the presence and absence of ascorbic acid, because of the previously demonstrated(1) favorable influence of reducing substances on the formation of the factor. Table I shows clearly that the CF-content of rat liver tissue, *in vitro,* was augmented by the addition of PGA. In the hepatic tissue of rats deficient in PGA, in contrast to liver slices from normal rats, very low values for CF were obtained. For this reason investigation of the inhibitory action of aminopterin on the synthesis of CF from PGA was facilitated by the use of PGA-deficient rats, rather than normal animals.

The data of Table I show that aminopterin markedly reduced the amount of CF formed from PGA by the liver slices of PGA-deficient rats. Since aminopterin is a potent inhibitor of the growth of *Leuconostoc citrovorum,* it was necessary to prove that the results observed actually were due to inhibition of hepatic synthesis of CF and were not to be accounted for by an antibacterial action on the microorganism subsequently used for assay. Absence of a significant effect of aminopterin, in the dilutions of the liver filtrates used for assay, is shown by the values for CF, designated in Table I by an asterisk, obtained when aminopterin was added to the slices at the end rather than at the beginning of the incubation period. These values agree closely with the corresponding values for CF found in the absence of aminopterin. The table shows also that the activity of aminopterin for the microorganism, unlike that of PGA, was not altered significantly by rat liver slices, since the CF activity found in control vessels containing no PGA did not differ appreciably from that of similar vessels in which 50 μg of aminopterin had been incubated with liver. Further, the inhibitory action of even higher concentrations of aminopterin on *Leuconostoc citrovorum,* grown in the presence of a limiting amount of CF, was not increased by a preliminary incubation of the inhibitor with liver slices.‡ The effect of ascorbic acid in augmenting the yield of CF, both in the absence and in the presence of PGA, is shown in Table I. The percentage inhibition of the production of CF caused by aminopterin, 50 μg per g of PGA-deficient liver tissue, was of the same order whether ascorbic acid was or was not present. In the amounts used, aminopterin, added to PGA-deficient slices, did not block completely the formation of CF from high concentrations of added PGA, although the yield was reduced by as much as 90%. On the other hand, addition of the antagonist (50 μg per g of tissue) to liver slices derived from normal rats abolished completely the extra CF formed from added synthetic PGA.

N^{10} - formyl - pteroylglutamate,§ "pteroyltriglutamate," and "pteroylheptaglutamate" were compared with pteroylglutamic acid, not only as precursors of CF, but also with regard to the effect of ascorbic acid and the inhibitory action of aminopterin on the derivation of CF from them. Each of these compounds yielded CF when incubated with liver slices from PGA-deficient rats and, in each case, the amount of CF synthesized in the tissue was increased in the presence of ascorbic acid. N^{10}-formyl-PGA was less active than PGA as a precursor of CF; however, the sample employed had not been subjected to rigid tests of purity and no particular significance is attached to the lower activity. "Pteroyltriglutamate" and "pteroylheptaglutamate" (crystalline vitamin B_c conjugate) were tested at concentrations approximately equimolar to PGA and showed about 50%

† A liver fraction is used in this laboratory as a constant reference standard; one unit of citrovorum factor is defined as that amount needed per 10 cc assay tube, to produce half-maximal turbidity in 20 hours at 37°.

‡ In the concentrations encountered, PGA does not influence the 20-hour growth of the organism. Studies of the stability of thymidine and of a naturally occurring material of unknown composition(7), under conditions which destroy CF, indicate that the accuracy of determination of the CF-values reported in this paper was not significantly influenced by these substances.

7. Nichol, C. A., and Welch, A. D., *Fed. Proc.,* 1950, v9, 367.

§ We are indebted to Dr. R. M. Anker who synthesized the N^{10}-formyl-pteroylglutamic acid in this laboratory according to the method of Gordon, *et al., J. Am. Chem. Soc.,* 1948, v70, 878.

TABLE II.
Inhibition by Aminopterin-Treatment, *in Vivo*, of the Conversion of Folic Acid to Citrovorum Factor by Liver Slices, *in Vitro*.

Additions to vessels containing liver slices*	Citrovorum factor activity—units/g of tissue. Source of tissue: PGA-deficient rats	Source of tissue: PGA-deficient, aminopterin-treated rats
None	80	40
PGA 10 μg	1610	60
PGA 100 μg	2600	110
Ascorbic acid, 10 mg	170	90
" " " " + PGA 10 μg	6960	170
" " " " + PGA 100 μg	11600	260

* The contents of the vessels and their manner of treatment are described in the text.

of the activity of PGA as precursors of CF, both in the presence and absence of ascorbic acid. Aminopterin inhibited the formation of CF from each of these forms of PGA. In the presence of 12.5 μg of aminopterin per g of liver slices, the yield of CF from PGA and from each of these derivatives of PGA was lowered to approximately 20% of that obtained without aminopterin. The effect of aminopterin on the ability of liver to convert PGA to CF also was studied by injecting aminopterin into PGA-deficient rats prior to the preparation of liver slices from these animals. Two groups of 8 rats each were selected from PGA-deficient animals which had not increased in body weight during the previous week. The rats of one group were given no additional treatment; each animal of the other group was injected intraperitoneally with 50 μg of aminopterin per day. The injected rats developed marked loss of body weight within 24 hours and severe diarrhea and secretion of porphyrin were observed on the second and third day of treatment. On the day following the third injection of aminopterin, 4 animals from each group were sacrificed for the comparison reported in Table II. Of the 4 remaining aminopterin-treated rats, 3 died on the third day and the last on the fourth day following the first injection. In the control group of PGA-deficient rats, body weight did not change appreciably and no deaths occurred during this time.

In contrast to the tissue from rats in the control group, liver slices from the injected rats were unable to form an appreciable amount of CF from PGA, even in the presence of ascorbic acid (Table II). Similarly, no CF was formed when formyl-PGA or the conjugates of PGA were incubated with liver slices from the aminopterin-treated rats, either in the presence or absence of ascorbic acid. Filtrates prepared from liver samples from rats which were injected with aminopterin did not inhibit growth of *Leuconostoc citrovorum* except at amounts per assay tube much greater than those required in the analyses. The prior treatment of the rats with aminopterin was more effective in reducing the yield of CF to be obtained from PGA added to the liver slices, than in those cases where the inhibitor was added *in vitro*.

Experiments in vivo. Sauberlich has shown that the urinary excretion of CF by rats is roughly proportional to the amount of PGA ingested(3). The inhibition by aminopterin of the conversion of PGA to CF observed in liver slice experiments, was tested *in vivo* by measuring the effect of aminopterin on the urinary excretion of CF by adult rats injected daily with PGA. Female rats (av wt, 150 g) were placed in individual metabolism cages equipped with paraffined-metal collecting funnels. These animals had been raised on a purified diet(1) containing 50 μg of PGA per 100 g and were fed this diet during the experimental period. Four groups were arranged, each containing three rats. Urine from each rat was collected under toluene during successive 48-hour periods and was kept refrigerated until assayed microbiologically with *Leuconostoc citrovorum* 8081. Urine from normal rats receiving 25 μg aminopterin per day was added to assay tubes

TABLE III.
Effect of Aminopterin on Urinary Excretion of Citrovorum Factor in Rats Receiving Pteroylglutamic Acid.

Group No.	Collection period, days	Treatment: PGA 2 mg/day	Treatment: Aminopterin, 25 μg/day	Treatment: Ascorbic acid, 50 μg/day	Citrovorum factor excreted per rat per 48 hr, units	Survival after initiation of aminopterin treatment, days
I	1-2	—	—	—	80	
(3 rats)	3-4	+	—	—	13800	
	5-6	+	+	—	4870	
	7-8	+	+	—	1770	
	9-10	+	+	—	1720	
	11-12	+	+	—	1420	
	13-14	+	+	—	1470	
	15-16	+	+	—	1660(2)*	12–14
II	1-2	—	—	—	80	
(3 rats)	3-4	+	—	+	52000	
	5-6	+	+	+	9840	
	7-8	+	+	+	6370	
	9-10	+	+	+	6250	
	11-12	+	+	+	2870	
	13-14	+	+	+	5720	
	15-16	+	+	+	2030(1)*	11–14
III	1-2	—	—	—	70	
(3 rats)	3-4	—	—	—	100	
	5-6	+	+	—	1030	
	7-8	+	+	—	900	
	9-10	+	+	—	1520	
	11-12	+	+	—	900	
	13-14	+	+	—	1210(2)*	8–11
IV	1-2	—	—	—	70	
(3 rats)	3-4	—	+	—	290	
	5-6	+	+	—	500	
	7-8	+	+	—	890	7–8

* Figures within parentheses represent the number of animals surviving the collection period indicated. CF values stated for all other periods represent the average of 3 urine samples.

containing limiting amounts of CF. It was found that the aminopterin excreted in the urine did not interfere appreciably with the growth of the organism at the dilutions used for assay. As indicated in Table III, daily doses of PGA, aminopterin, and freshly neutralized ascorbic acid were administered intraperitoneally. Group II duplicated the treatment of Group I, except that ascorbic acid was given along with PGA, since ascorbic acid has been found to increase the urinary excretion of CF by rats and human subjects receiving PGA(8). Groups I, III, and IV differed only in the times when administration of PGA and aminopterin were begun. Group I was given PGA during a 48-hour period prior to the initiation of treatment with aminopterin; Group IV received aminopterin during a 48-hour period prior to the initiation of treatment with PGA; in Group III PGA and aminopterin were administered simultaneously.

The data obtained from this experiment are presented in Table III. Normal rats fed a purified diet(1) containing succinylsulfathiazole (2%) excreted from 60 to 100 units of CF per 48 hours (all groups). Administration of PGA (2.0 mg per day) increased the CF content of the urine by about 150-fold (Group I). An even greater increase in the excretion of CF followed treatment with both PGA and ascorbic acid (Group II). When aminopterin was given simultaneously with PGA the amount of CF excreted was markedly reduced (1030 units, Group III,

8. Unpublished data from this laboratory and Welch, A. D., Trans. Assoc. Amer. Physicians, 1950, in press.

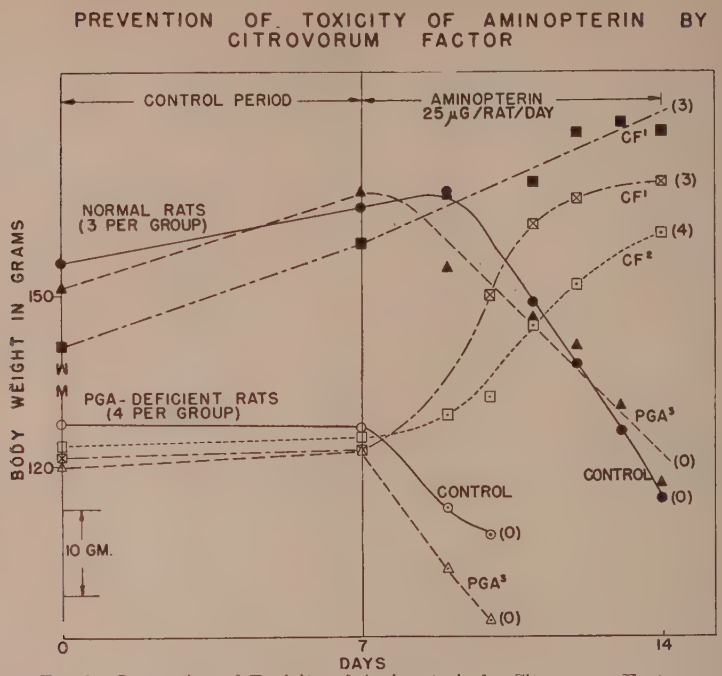

FIG. 1. Prevention of Toxicity of Aminopterin by Citrovorum Factor. Figures enclosed in parentheses show the number of animals surviving on the day indicated.

1 500,000 units of CF (3 mg of concentrate) were inj. daily after 7th day.
2 250,000 units of CF (1.5 mg of concentrate) were inj. daily after 7th day.
3 5 mg of pteroylglutamic acid were inj. daily after the 7th day.

as compared with 13,800 units, Group I). Pre-treatment of rats with aminopterin for 2 days prior to initiation of PGA injections reduced further the urinary elimination of CF formed from PGA (Group IV). In the rats which at first were injected only with aminopterin (Group IV) there was a suggestion of a slight increase in the elimination of CF; this will be discussed subsequently. The large amounts of CF excreted by the rats first given PGA or PGA plus ascorbic acid decreased markedly when additional injections of aminopterin were begun. During continued treatment with PGA and aminopterin, some CF was formed, as evidenced by the excretion data. However, all rats died within 14 days following initiation of treatment with aminopterin. Administration of PGA prior to treatment with aminopterin increased the survival time. Since aminopterin inhibits the metabolic alteration of PGA, the product of this reaction might be capable of competing more effectively than PGA with this antagonist. Accordingly, the ability of concentrates of CF to prevent the toxicity of aminopterin was tested in PGA-deficient and in normal rats. In these experiments young female rats were given a purified diet(1) during a period of 6 weeks. To the diet of one group ("normal"), PGA was added at a level of 50 μg per 100 g. During a control period of one week, the PGA-deficient rats showed no significant change in body weight, while the "normal" rats continued to gain at a uniform rate, as is shown in Fig. 1. During the period of treatment, all rats were injected with aminopterin (25 μg per day) intraperitoneally. The control groups received no additional treatment. Other groups were given injections of PGA (5 mg per day) or of a concentrate of CF supplied by the Lederle Laboratories; this concentrate was

tested at two levels in the PGA-deficient rats (1.5 mg per day, providing 250,000 units, and 3.0 mg per day, providing 500,000 units of CF). In the "normal" rats the CF concentrate was administered intraperitoneally at a level of 500,000 units per day.

PGA failed to protect the rats from the lethal action of aminopterin. Although the "normal" rats were somewhat more resistant to the analogue than were the PGA-deficient animals, none survived the eighth day of injection with aminopterin. Comparable treatment of the PGA-deficient rats led to death by the fourth day of treatment. In contrast to the ineffectiveness of PGA, the concentrate of CF exerted a marked protective action against the toxic effects of aminopterin (see Fig. 1). "Normal" rats given the concentrate, together with aminopterin, maintained the same rate of growth that was observed during the control period. Of the PGA-deficient rats which were given the concentrate of CF, as well as aminopterin, only one animal died (on the third day of treatment). The other rats maintained a remarkable rate of growth. The number of animals used in this preliminary experiment was limited by the small amount of CF concentrate available. Consequently, no significance is attached to the difference in rate of growth between the groups receiving the two levels of CF concentrate. However, only those animals which received this concentrate survived and these rats remained healthy following the cessation of treatment (see Fig. 1).

Discussion. The data presented show clearly that aminopterin interferes, both *in vitro* and *in vivo,* with the conversion of pteroylglutamic acid to the factor(s) which promotes rapid growth of *Leuconostoc citrovorum* 8081. The product of the metabolic alteration of PGA, citrovorum factor, not only is capable of preventing the toxicity of aminopterin, but also is a biologically active derivative of PGA, as demonstrated by the growth response of PGA-deficient rats which received aminopterin and a concentrate of CF concurrently. Inhibition of the conversion of PGA to CF was more complete in liver slices from rats which were injected with aminopterin than when the antagonist was added directly to the tissue *in vitro.* Administration of aminopterin to the intact animal apparently allows greater opportunity for saturation of the enzymes responsible for the conversion of PGA to CF. Whether these enzymes are widely distributed throughout the tissues or are restricted to the liver has not yet been determined. The failure of massive doses of PGA to prevent the toxic effects of aminopterin has been demonstrated in several species(4,5,9,10,11). This antagonist appears to have much greater affinity than PGA for the enzymes which accomplish the conversion of PGA to CF. Sufficiently high concentrations of PGA in the tissues should limit the ability of the antagonist to combine with these enzymes, but in most cases this concentration apparently is not readily attainable. Thus, PGA at a level of 10 mg per 100 g of diet failed to counteract the lethal effect of aminopterin (100 μg per 100 g in mice)(4); recently, however, Ershoff, Hoffstadt and McWilliams(12) reported positive protection in this species when a ratio of PGA to aminopterin of 500:1 was used. Also, Hertz and Tullner(13) observed that inhibition by aminopterin of the estrogen-induced growth in the female genital tract of estradiol-treated ovariectomized rats and stilbesterol-treated chicks could be counteracted at PGA to inhibitor ratios of 300:1 or 400:1.

The action of aminopterin cannot be explained solely on the basis of an interference with the metabolic alteration of PGA. Studies of the effect of this antagonist on certain bacterial species, particularly *Leuconostoc citrovorum* 8081, indicate that aminopterin interferes with the utilization of CF(6,14,15). Data presented in this paper support this

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conclusion. Rats given injections of both PGA and aminopterin excreted from 1000 to 2000 units of CF in the period immediately preceding death (Table III). This amount of CF far exceeds that excreted by "normal" rats fed a diet adequate in PGA (50 μg per 100 g), yet this amount of CF excreted in the urine reflects tissue concentrations incapable of reversing the toxic effects of aminopterin. However, larger amounts of CF effectively counteracted the antagonist (Fig. 1). Presumably aminopterin competes with CF for combination with an apoenzyme or enzyme with which CF normally reacts. Preliminary evidence suggests that the activity of 1 μg of pure CF may lie between 1000(16) and 10,000 units(15). Since 250,000 units of CF protected rats completely against the toxic effects of 25 μg of aminopterin, it may be suggested that, on a weight basis, the ratio of CF to aminopterin required to prevent the effects of the antagonist is less than 1:1 or 10:1. Recent studies by Broquist, *et al.*(15), using mice, also have shown that concentrates of CF can prevent the toxic effects of aminopterin. Whether CF can reverse the toxicity of aminopterin when treatment is delayed until the toxic effects of the drug are strongly manifested is under investigation.

The survival time of rats injected with aminopterin was increased by prior treatment with PGA (Table III). This effect may be due to saturation of the tissues with CF so that depletion of CF and inhibition of its functions by aminopterin require a longer time. Also, CF may be formed from certain precursors in the tissue by reactions which are not inhibited by aminopterin. This is indicated by data in Table I which show that, in normal liver slices, aminopterin did not influence significantly the amount of CF which was derived from tissue-precursors. Also, the additional CF formed from tissue-precursors in the presence of ascorbic acid was not blocked by aminopterin. However, under the same conditions, the conversion of added PGA to CF, which also is increased by ascorbic acid, apparently was inhibited completely by the analogue. Conceivably, these findings might signify that, in the conversion of PGA to materials with high activity for *Leuconostoc citrovorum,* at least two steps are involved. Only the first of these may be blocked by aminopterin, while the second step could involve a reduction facilitated by ascorbic acid but not interfered with by aminopterin. According to this view, there may be present in normal rat liver a compound with a structure intermediate between those of PGA and CF, and on which the effect of ascorbic acid is exerted.

Swendseid, *et al.*(17), observed a small increase in the urinary elimination of CF following administration of aminopterin to human subjects. A similar observation was made in each rat injected with aminopterin alone (Table III). This may reflect a displacement of CF from tissue complexes by the antagonist.

Information derived from these studies indicates that PGA is converted metabolically into a biologically more effective compound and provides an explanation for the biochemical mechanism of action of a very potent poison and for the failure of PGA effectively to counteract its toxicity. Further, and in confirmation of the work of Broquist, *et al.* (15) in mice, a means is afforded of counteracting the easily developed toxic action of aminopterin, a compound of considerable utility in the therapy of certain types of acute leukemia in children.

Summary. Rat liver slices form from synthetic folic acid (PGA) a factor (CF) utilized for growth by *Leuconostoc citrovorum;* this conversion is inhibited by aminopterin. In rats given PGA, a similar inhibition of the synthesis of CF is shown by a marked decrease in the urinary excretion of CF following the administration of aminopterin. The antagonist not only prevents the metabolic altera-

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tion of PGA but also competes with the product (CF) derived from PGA. The lethal action in rats of aminopterin, 25 μg daily, is not prevented by PGA, but the daily administration of 250,000 units of CF completely counteracts the toxic effects of the antagonist. That CF is a biologically active derivative of PGA is shown by the fact that rats in which growth is arrested by PGA-deficiency, and which due to aminopterin are refractory to PGA, grow remarkably when given concentrates of the citrovorum factor.

We are especially appreciative of the invaluable technical assistance given by Marilyn F. Wilson and Caroline A. McKalen.

Pteroylglutamic acid, "pteroyltriglutamate" and some of the concentrates of citrovorum factor were furnished by the Lederle Laboratories Division of America Cyanamid Co., through the courtesy of T. H. Jukes; other concentrates of the citrovorum factor were prepared by R. M. Anker and J. W. Boehne, III, of this laboratory. Crystalline vitamin B_c conjugate was a gift from J. J. Pfiffner of Parke, Davis & Co., and succinylsulfathiazole was provided by Sharp and Dohme, Inc., through the courtesy of W. A. Feirer.

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Tocopherol Requirements of Rats by Means of the Hemolysis Test.* (17923)

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It has been found that in rats deficient in vitamin E, injection of alloxan or its reduction products, alloxatin and dialuric acid, is followed within a few minutes by hemolysis so severe that in some cases the cell volume is reduced from the normal value of over 50% to 10% or less(1,2). The same reaction may be produced in washed red blood cells with dialuric acid(2,3). Alloxan itself is not hemolytic *in vitro* so it may be presumed that its action *in vivo* depends on reduction to dialuric acid by sulfhydril or other reducing compounds. *In vivo* or *in vitro* the blood cells of animals receiving adequate amounts of tocopherol are completely protected against the hemolyzing effect of dialuric acid. The *in vitro* hemolysis test requires only a few drops of blood and has proved to be a convenient method of studying some aspects of the requirement and utilization of tocopherol in the rat.

Experimental. The rats used in these studies were females of the Sprague-Dawley strain weighing about 70 g when they were received. Depletion studies were not begun until the rats weighed about 110 g. Until this time they were given the Steenbock stock diet(4) supplemented with milk and cabbage, and the animals referred to as "stock" or "normal" received this ration throughout the whole experimental period. The vitamin E-deficient diet contained casein (General Biochemicals, vitamin test), 20; lard, 38; cod liver oil, 2; salt mixture (U.S.P. No. 2), 4; sugar, 36; and in some instances yeast, 5 (replacing an equal weight of sugar). Each animal received a daily supplement of 20γ thiamine chloride, 100γ calcium pantothenate, 20γ pyridoxine, and 25γ riboflavin. In most of the experiments the vitamin E supplement was given in the form of mixed natural tocopherols (Distillation Products) dissolved in cottonseed oil (when massive doses were given) or in peanut oil. In one study a synthetic DL-alpha tocopherol (Merck) was used.

For the hemolysis test 8 or 10 drops of blood

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were collected from the tail into 1 or 2 ml of a solution containing 0.9% of sodium chloride and 1% of sodium citrate in a graduated centrifuge tube. The blood was centrifuged, the supernatant fluid removed and 0.9% sodium chloride added to make a 5% suspension of the blood cells. To 0.25 ml of this suspension in a small tube were added 0.18 ml of phosphate buffer (25 ml of 0.2 M potassium monophosphate, 19.7 ml of 0.2 M sodium hydroxide, with water to make 100 ml) and 0.07 ml of a 0.1% solution of dialuric acid in the buffer. The tubes were incubated for 15 min. at 37°C, after which they were kept at room temperature and the time required for the cell suspension to become clear noted. A control tube without dialuric acid was run with the test sample. When the blood cells of animals which had received the deficient diet for several weeks were tested, there was complete hemolysis within half an hour. If the cells were partially protected either because of a shorter depletion period or an inadequate intake of tocopherol, hemolysis occurred, but more slowly. In the following discussion hemolysis (or tocopherol deficiency) has been classified as slight if 4 or more hours were required for the suspension of red blood cells to become completely clear, moderate if there was complete lysis in 1 to 4 hours. The blood of a tocopherol-treated animal was run as a control with each test. No hemolysis was ever observed in these cells.

Results. Rate of tocopherol depletion. The red blood cells of animals receiving the stock ration usually showed no hemolysis when treated with dialuric acid. In a few cases a faint trace was observed. After these rats had received the tocopherol-deficient diet for only 3 days there was definite, though slight, hemolysis; in 7 days it had increased considerably, and after 10 days almost the maximum rate of hemolysis had been reached. In marked contrast to the rapid tocopherol depletion in these animals was the response of a group which, for a considerable period, had been given 3 mg of tocopherol daily as a supplement to the deficient diet, and then kept on the deficient diet alone. Four of these rats which had received the supplement

TABLE I.

Effect of Tocopherol Administration to Rats on the Sensitivity of the Red Blood Cells to Hemolysis by Dialuric Acid. (10 rats in each group).

DL-alpha tocopherol, mg/day	No. of rats protected: Time after addition of dialuric acid to R.B.C. 1 hr	2 hr	4 hr
.00	0		
.10	2	0	
.14	4	1	0
.20	8	4	0
.28	10	10	8

for about 100 days showed only slight hemolysis after 118 days without tocopherol. Two animals which had received tocopherol for 44 days showed only this same slight sensitivity to dialuric acid after deprivation for 91 days. The tendency to hemolysis increased very slowly after this time. Three animals died before they were completely depleted. Nine months after treatment was discontinued hemolysis had reached the maximum rate in two of the remaining animals while the blood of the third was still partially protected.

Protective and curative doses of tocopherol. To determine how much tocopherol was required to protect the red cells, rats were transferred to the vitamin E-deficient ration and groups of 2 given 0.05, 0.10, 0.20, and 0.40 mg of mixed natural tocopherols daily. When the animals had received the experimental ration for 2 weeks the blood was tested and only with the highest dose of tocopherol was hemolysis completely prevented. In a more extended study, groups of 10 animals were given 0.10, 0.14, 0.20 and 0.28 mg per day of DL-alpha tocopherol. The results are shown in Table I. There is good proportionality between the dose and the number of rats protected. The highest dose given was a little less than optimal since 2 animals were not completely protected. If partial rather than complete protection of the red cells had been tabulated, a somewhat greater protective effect of the lower doses of tocopherol would have been demonstrated. In the control group the cells of every rat were completely hemolyzed in less than one hour. Of the animals receiving 0.10 mg of tocopherol

only 2 showed complete hemolysis in one hour and with the higher doses, in no animal was there more than partial hemolysis in this period. The deficiency of tocopherol in the erythrocytes could be rapidly overcome. Two rats which had received the deficient ration for one month were given a single dose of 1.5 mg of natural tocopherols. The red cells were completely resistant to hemolysis the next day, and some protective effect persisted for about 10 days. A dose of 0.5 mg was inadequate for demonstration of any protective effect in 24 hours. When the dose was repeated for 3 days there was some protection but it was never complete and disappeared as soon as therapy was discontinued.

Tocopherol in the blood of newborn rats. Transfer of tocopherol across the placenta is known to be poor, and it was of interest to determine the response of the erythrocytes of newborn rats to dialuric acid. Sufficient blood for a determination could be obtained from 1 or 2 animals by decapitation. Litters of 4 females receiving the stock ration were studied. Two of these litters were born during the day and the blood of the young rats was tested within an hour. Hemolysis was complete in less than an hour. Hemolysis was slower in 2 litters born during the night and tested the following morning, but a moderate degree of tocopherol deficiency was still indicated. Three of the litters were completely protected against hemolysis on the second day of life while the blood of the rats in the fourth litter was still sensitive to dialuric acid. The animals of this litter were in poor condition and probably had received no nourishment. Four females were given 5 mg a day of tocopherol throughout the gestation period. There was no hemolysis in any of the young tested. Unfortunately, all of the litters were born at night and the possibility that they might have been deficient in tocopherol at birth was not entirely precluded. In order to make sure that the difference between the 2 groups was not due to any post-natal influences, caesarian section was performed at about the twentieth day of pregnancy on 2 females receiving the supplement of tocopherol and 2 females given the unsupplemented diet. There was no hemolysis in the fetuses of the first group, while in the second, hemolysis was observed as with the newborn litters.

Plasma tocopherol determinations. In order to compare the degree of tocopherol deficiency measured by susceptibility to hemolysis with deficiencies described in the literature, tocopherol was determined in the plasma of stock, tocopherol - deficient and tocopherol - treated animals. Some of the deficient animals and all of the tocopherol-treated animals had received an injection of alloxan or dialuric acid one or 2 months previously, from which they had recovered without serious after-effects. The method of Hines and Mattill(5) utilizing the colorimetric procedure devised by Emmerie and Engel(6) was used. A simple, one-step extraction which Quaife and Harris (7) found satisfactory for plasma was substituted for the more laborious procedure necessary with muscle and liver. The tocopherol-treated animals received 3 mg of tocopherol a day as a supplement to the deficient diet for about 2 months and then were returned to the stock diet and given a single dose of 9 mg of tocopherol once a week. The average value of plasma tocopherol in this group was of the same order as the normal human value reported by a number of workers: 1.0 ± 0.08 mg per 100 ml. The plasma of the rats receiving the stock ration contained less than half this amount of tocopherol, 0.38 ± 0.02 mg per 100 ml while the level in the group on the deficient diet, 0.27 ± 0.04 mg per 100 ml was not a great deal lower than that of the stock animals.

Discussion. Susceptibility to hemolysis by dialuric acid differs from other manifestations of vitamin E deficiency in the rat in the short time required for its demonstration in relatively mature animals. Signs of deficiency in the blood cells could be detected in rats 2 to 3 months old after only a few days on the tocopherol-deficient diet, and the

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maximum rate of hemolysis was reached within 2 weeks. Approximately 3 mg of DL-alpha tocopherol per kg of body weight per day was required to protect the red cells. This is several times the average minimum prophylactic dose for prevention of sterility or muscular dystrophy(8).

In comparing the animals receiving the stock ration with those given supplements of tocopherol or a tocopherol-deficient diet, it is disturbing to see how close these presumably normal animals were to the deficient level. This is apparent not only from the plasma tocopherol values but from the depletion studies. During the period of treatment rats given supplements of tocopherol stored an amount of it sufficient to maintain them for 6 to 9 months above the deficient level as measured by susceptibility to hemolysis. The normal animals were young when they were put on experiment and one would not expect a large reserve of tocopherol. The fact that they were depleted within a week indicated that there was practically no reserve. A similar observation was made by Hines and Mattill(5).

There have been a number of reports in the literature as to the relative deficiency of the newborn in tocopherol(9,10,11). If, as our experiments have suggested, tocopherol serves as a protective agent against damage to erythrocytes (and perhaps other tissues) by toxic substances, the fetus is in a peculiarly defenseless position, and may be damaged by conditions which would not affect the mother.

In our experiments the young rats were completely protected against the hemolyzing effect of dialuric acid within one day. Mason (9) observed that in sucklings 24 to 48 hours old the tocopherol content was increased to the adult level. It has been found in cows(12) and ewes(13) as well as in human beings(14) that colostrum contains several times as much tocopherol as late milk. The tocopherol deficiency of the red blood cells of the infant rats could be prevented by supplementing the ration of the mother with tocopherol during the gestation period. Whiting and Loosli(13) reported similarly that the tocopherol content of the blood plasma of newborn sheep and goats was increased by prepartum supplementation with tocopherol. Parrish, *et al.*(15) on the contrary, found little effect of prepartum supplementation of the dams with tocopherol on the plasma tocopherol level in calves.

Of the various manifestations of vitamin E deficiency, fetal resorption has proved most satisfactory for bioassay. However, the method is cumbersome and results from different laboratories are not entirely consistent. The studies of the treatment and prevention of tocopherol deficiency which have been described indicate that the change in the red blood cell may be utilized in the assay of vitamin E. The procedure has the advantages of speed and simplicity. Whether it has the necessary precision will be determined by further tests.

Summary. The sensitivity of the red blood cells of tocopherol-deficient rats to the hemolyzing action of dialuric acid *in vitro* has been applied to the study of tocopherol requirements. Rats which had received the stock diet until they were 2 to 3 months old showed signs of deficiency after only a few days on a tocopherol-deficient diet. Animals which had received large supplements of tocopherol were protected many months after it was discontinued. About 3 mg of DL-*a*-tocopherol per day per kg of body weight was required for protection of the erythrocytes. The newborn young of rats which had received the stock ration were susceptible to the hemolyzing action of dialuric acid. If the

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females were given a supplement of tocopherol throughout the gestation period the blood cells of the young rats were protected. The possible application of this hemolysis method to the bioassay of vitamin E has been discussed.

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Experimental Control of Serum Calcium Levels *in Vivo*.* (17924)

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The constancy of the total calcium level of the blood is the result of the interplay of numerous factors which include calcium intake, absorption and excretion, Vitamin D economy, parathyroid gland function, and blood phosphate, bicarbonate, protein and pH levels(1). The relations which determine the distribution of the total blood calcium between the ionic and the bound forms are largely unknown. It is generally accepted that the ionic calcium in the blood is the physiologically active form. Were it possible to control the ionic blood-calcium levels by exogenous means further insight into the relationships and mechanisms which govern calcium levels and distribution might be obtained. Soluble oxalates lower blood calcium ions by precipitation of insoluble calcium oxalate. Citrates, tartrates, phthalates, fluorides, polyphosphates, glycine, and other anions lower blood calcium ion by the formation of soluble nonionic complexes(2). The characteristic physiological sequelae attributable to hypocalcemia follow the administration of these agents. However, the use of these materials for quantitative control of calcium ion *in vivo* is subject to certain experimental difficulties. Ethylenediamine tetra-acetic acid (E.D.T.A.A.) forms soluble nonionized complexes with many bivalent cations(3) and behaves as a chelating agent. Dyckerhoff *et al.*(4) have reported that E.D.T.A.A. inhibits blood coagulation *in vitro* probably as the result of the removal of calcium ion by complex formation. At physiological pH we have found that in human or rabbit serum the complex of calcium ion with E.D.T.A.A. forms stoichiometrically. Calcium ion bound in E.D.T.A.A. complex is not precipitated by the addition of oxalates and hence the total remaining non-complexed calcium which may consist of both ionic and protein bound calcium can be determined by oxalate precipitation. We have demonstrated this by numerous *in vitro* experiments. That the same combination occurs *in vivo* is illustrated in Fig. 1 where the physiological action of E.D.T.A.A. was shown to be the consequence of its combination with calcium ion. Rapid (20 sec.) intravenous injection into the marginal ear vein of rabbits of E.D.T.A.A. as the neutral sodium salt, 100 mg/kg, resulted in immediate lowering of the available calcium level with consequent death by hypocalcemic tetany. When the calcium chelation of E.D.T.A.A. has been saturated by admixture with stoichiometric quantities of calcium in the form of its chloride or gluconogluconate salt prior to injection, the previously demonstrated toxic effects did not occur (Fig. 1). A second injection of the neutral sodium salt of E.D.T.A.A. 100 mg/kg, then caused

* Supported by grants from the National Institutes of Health, the Geschickter Fund for Medical Research, and the Bersworth Chemical Co.

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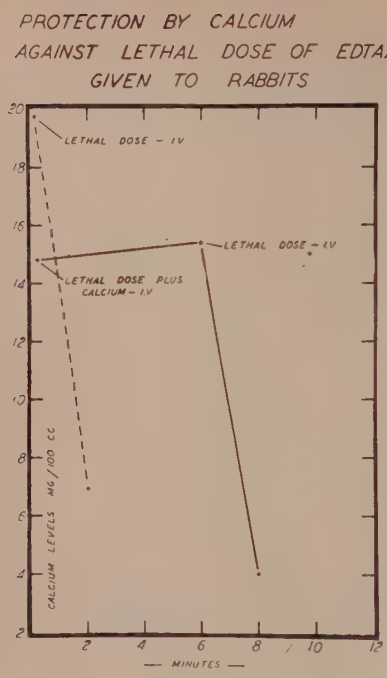

FIG. 1.

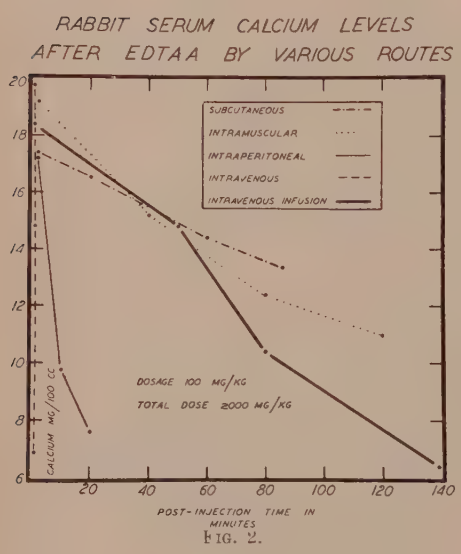

FIG. 2.

death of the rabbit in the typical manner. In numerous experiments it has been demonstrated that complete recovery from the effects of E.D.T.A.A. could be brought about by intravenous or intracardiac administration of calcium salts at any time prior to death. The rapidity with which the calcium level is lowered is a function of the mode of administration of the chelating agent (Fig. 2) and the rate at which it is injected as well as the dose. Intravenous, intraperitoneal, intramuscular and subcutaneous injection, as might be anticipated, resulted in successively diminishing rates of lowering of the available calcium serum levels. By slow intravenous drip it was possible to inject 2,000 mg per kilo of E.D.T.A.A. over a period of 3 hours before the serum calcium fell to a fatal level. By controlling the rates and amounts of chelate given it was possible to lower the available calcium serum level and maintain this level by balancing the complex formation against replenishment of blood calcium from the mobile reserves of the animal. That E.D.T.A.A. may be absorbed through the skin is illustrated in Fig. 3. Daily applications of a 5% water-soluble ointment to 2 human subjects resulted in a gradual fall of the serum calcium to 8 mg per 100 cc in a period of 4 days. Intravenous administration of calcium gluconogluconate resulted in the re-establishment of normal calcium levels.

Magnesium ion may have a regulatory action on calcium metabolism as has been suggested by Mendel and Benedict(5) and Becka(6). More direct evidence for this

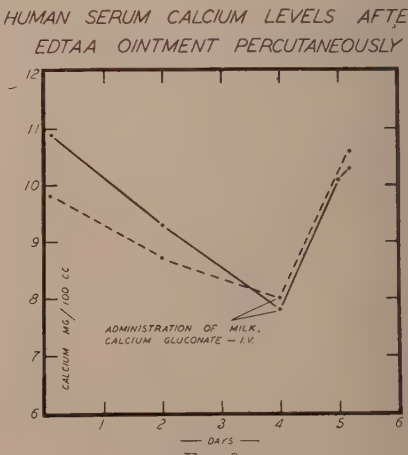

FIG. 3.

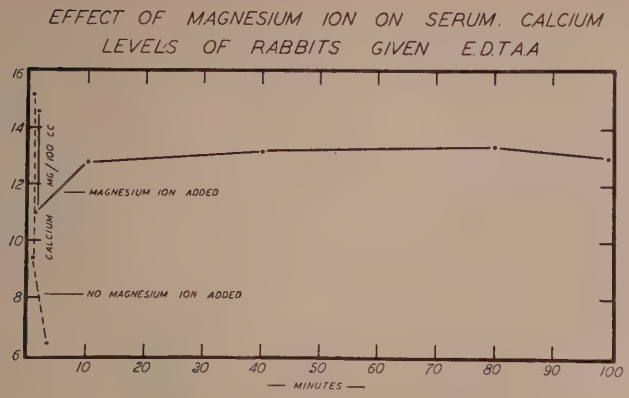

FIG. 4.

function than hitherto available is presented in Fig. 4. It can be noted that simultaneous administration of magnesium ion with an ordinarily lethal dose of E.D.T.A.A. resulted in a rapid replenishment of the blood calcium levels. This effect was noted whether the magnesium was pre-added to the E.D.T.A.A. solution with formation of the magnesium complex of E.D.T.A.A. prior to injection or if magnesium ion in the form of the sulfate was given simultaneously into the opposite ear vein of the rabbit or was injected directly into the heart simultaneously with the intravenous injection of E.D.T.A.A. in the ear vein. That the result was not due to combination of E.D.T.A.A. with magnesium ion rather than calcium ion has been indicated by the demonstration that the complex of calcium ion by E.D.T.A.A. as measured by the precipitation of unbound calcium by oxalate did not change in the presence of magnesium ion in the system when tested *in vitro*. Confirmation of this work was reported in a study by physical means of the preferential complex formation of E.D.T.A.A. with various bivalent cations(7). In the physiological pH range E.D.T.A.A. combines with calcium ion preferentially over magnesium ion.

Summary. The ability of ethylenediamine tetra-acetic acid to form undissociated calcium complexes at physiological pH has been utilized as a tool to regulate available serum calcium levels *in vivo*. Further evidence has been presented for the regulatory action of magnesium ion on serum calcium levels.

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Effect of Physical Factors on Radiosodium Clearance from Subcutaneous and Intramuscular Sites in Animals.* (17925)

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This experimental study was based upon the recent work of Kety(1,2,3) and Elkin *et*

* Presented before the American Physiological Society, April 21, 1950.

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2. Kety, S. S., *Am. Ht. J.*, 1949, v38, 321.

al.(4) in which the rate of clearance of radiosodium from intramuscular injection sites has been used to measure the effective circulation of a tissue. Stone and Miller(5) established that lymphatic drainage does not have a significant role in the intramuscular clearance of Na^{24}, *nor* does the radioactive material diffuse along intramuscular planes. Since these reports indicate that the clearance of radiosodium may be of investigative and diagnostic aid this study was undertaken to determine: 1. The resting clearance rates of radiosodium from intramuscular and subcutaneous injection sites. 2. The effect of alterations in local temperature, abdominal pressure and hyaluronidase on the clearance rates.

Methods and materials. Radiosodium was prepared in the form of sodium chloride by neutron bombardment in the cyclotron of the Sarah Mellon Scaife Radiation Laboratory of the University of Pittsburgh. Approximately 5.0 microcuries of Na^{24} in physiological saline solution in the volume of 0.25 to 2.0 cc were used for injection into the hamstring muscles of 25 dogs of approximately 12 kg weight under pentobarbital sodium anesthesia. Subcutaneous injections were similarly made into the area on the medial aspect of the thigh in 10 dogs. Using a lead shielded Geiger-Muller tube, coupled to an autoscaler, the decline of activity as expressed in counts per minute was measured by multiple determinations at intervals of 2 minutes. Decline of activity was expressed as a slope or clearance constant after the method of Kety(2) using the formula,

$$K = \frac{\text{Log } C_1 - \text{Log } C_2}{0.4343\ (T_2 - T_1)}.$$

K = Clearance constant of Na^{24}.
C_1 = Activity as counts per minute at beginning time or T_1.
C_2 = Activity as counts per minute at end time or T_2.
0.4343 = Log e.

The clearance of radiosodium, as the decline in activity measured at the injection site, was also conveniently expressed as the percentage of activity remaining per unit time considering the activity in counts per minute at initial time as 100%.

Following a preliminary 10 minute control clearance, determination of the effect of the application of radiant heat from an infrared source 18 inches from the skin, producing a surface temperature of 55°C, was studied in 6 dogs. The effect of local application of an ice-salt mixture to the site of injection was similarly studied in 8 dogs and the effect of applications of an abdominal cuff inflated to produce interruption of venous return was observed in 5 dogs. The effect of the addition of 250 viscosity units of hyaluronidase to subcutaneous injections was also determined. In a series of 6 rabbits under pentobarbital sodium anesthesia, alterations in clearance rates at intramuscular radiosodium injection sites were studied by separate and simultaneous clamping of the common iliac artery and vein in order to evaluate the comparative role of the artery and vein in altering clearance rates.

Fig. 1 shows the clearance of radiosodium from an intramuscular site observed for a total time of 161 minutes plotted semi-logarithmically. Because of the short period of observation with respect to the 14.8 hour half life of Na^{24} the results are not corrected for the decay of radiosodium over this period

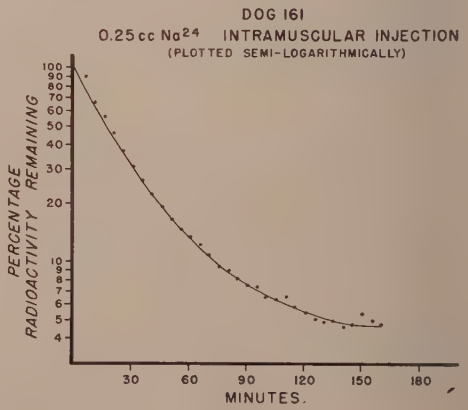

FIG. 1.

3. Wechsler, R. L., Sokoloff, L., and Kety, S. S., *Fed. Proc.*, 1950, v9, 133.

4. Elkin, D. C., Cooper, F. W., Jr., Rohrer, R. H., Miller, W. B., Jr., Shea, P. C., Jr., and Dennis, E. W., *Surg., Gynec. and Obst.*, 1948, v87, 1.

5. Stone, P. W., and Miller, W. B., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 529.

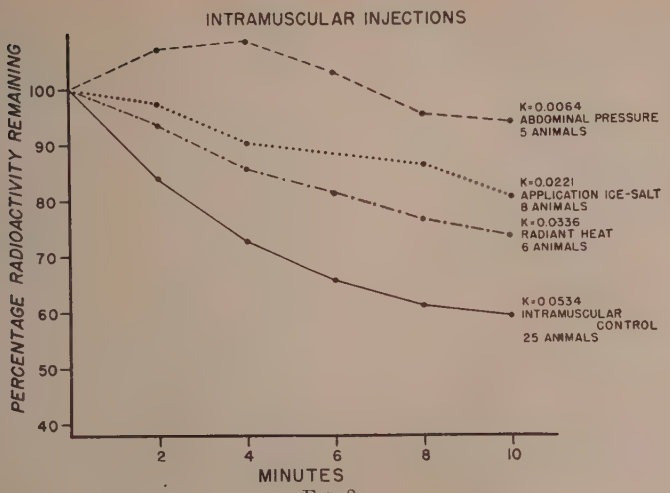

FIG. 2.

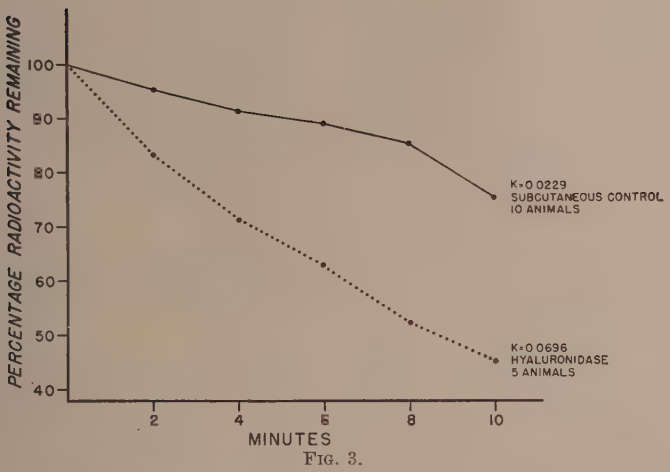

FIG. 3.

but demonstrate that over a relatively long time the rate of intramuscular clearance is not a true straight line function. In none of the intramuscular experiments did the data produce a straight line when plotted semi-logarithmically.

Fig. 2 summarizes the averages of the control and experimental results over a 10 minute observation period chosen for comparison purposes. The average intramuscular control clearance rate in 25 studies for a 10 minute period can be expressed by a clearance constant of 0.0534, or 59.4% activity remaining. Similarly, for 6 studies of radiant heat applied to the intramuscular injection site the clearance constant was 0.0336 or 73.5% activity remaining. The average clearance constant in 8 animals with the ice-salt application to the intramuscular injection site was 0.0221 or 80.5% activity remaining; with interruption of venous return by the abdominal cuff a clearance constant was obtained of 0.0064 or 94.0% activity remaining. All determinations showed a gradual decline of activity at the site of injection except during the application of pressure in which a preliminary increase in activity at the site of injection was observed.

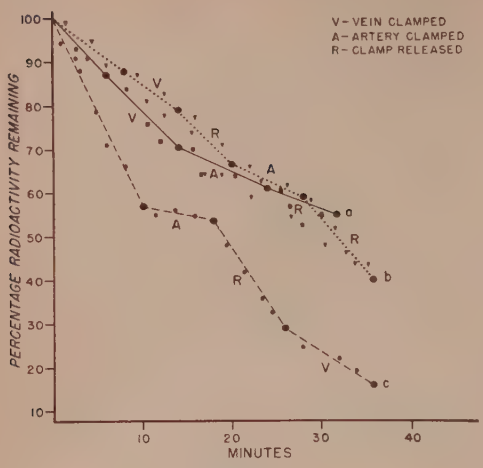

FIG. 4.

Fig. 3 demonstrates that with subcutaneous injection, the average control clearance constant was 0.0229 or 75.8% activity remaining. The addition of 250 viscosity units of hyaluronidase to the isotope solutions produced a marked increase to a clearance constant of 0.0696 or 45.6% activity remaining in 10 minutes.

In Fig. 4 the rate of intramuscular clearance in 3 anesthetized rabbits expressed in per cent activity remaining per unit time demonstrates the comparative effect on the clearance rate by clamping (a) the common iliac vein followed by clamping of the common iliac artery, (b) by clamping the vein followed by release of the vein and then clamping the artery, and (c) clamping the artery followed by release of the artery and then clamping the vein. Decreases in clearance rates in all instances were far more marked with arterial clamping than with clamping of the vein. In Fig. 4a and 4b there is no definite change in clearance rate with the vein clamped.

In these experiments comparison of the control intramuscular clearance rates with body weight, the depth of anesthesia, the volume of the radioactive solutions used, and the total amount of radioactivity per injection showed no discernible correlation.

Discussion. This method provides a technic to determine the gross effective circulation of a tissue and to quantitate that flow by a standard clearance procedure. The technic is simple and the radiation hazards are minimal. Since the amounts of radioactivity injected are less than 5.0 μc a very small quantity of radiation is delivered at the site of injection even when the tissue clearance rate is slow. The total body exposure after the Na^{24} enters into the general circulation is 5% of that used by Smith and Quimby(6). Our results plotted semi-logarithmically (Fig. 1) failed to demonstrate a straight line function for the removal of radiosodium particularly after the first 10 minutes of study. Since the formula suggested by Kety for the calculation of clearance constants was based upon an exponential curve the slope of which should yield a straight line we prefer the simpler calculation of the percentage of activity remaining per unit time. This figure readily serves to indicate the rate of clearance and to quantitate the effective blood flow.

The results for the control intramuscular clearance of radiosodium of 59.4% activity remaining in 10 minutes compared favorably with those of approximately 5% removed per minute reported by others(2,4). The application of freezing mixtures to the site of injection produced a definite decrease in effective blood flow. However, the use of radiant heat to the point of producing visible hyperemia decreased the clearance of radiosodium. This result which is not in agreement with the generally accepted opinion of the effect of local heat, again demonstrates that the status of skin vessel dilation is an inadequate criterion of effective circulation. Abdominal pressure to the point of stopping venous return gave an average of 94% activity remaining after a 10 minute application. The early, small increase in radioactivity at the site of observation may represent a transitory backflow and leakage from vessel wall with the buildup of a pressure head before accessory channels aid in the clearance of the radiosodium.

The subcutaneous clearance rate of 75.8%

6. Smith, B. C., and Quimby, E. H., *Radiology*, 1945, v45, 335.

activity remaining in 10 minutes, showed a marked increase with addition of hyaluronidase to 45.6% activity remaining in 10 minutes. The preliminary study suggests definite advantages to the subcutaneous route over the intramuscular particularly for clinical study of effective blood flow because of the ease of injection, deviation of subsequent clearance rates and the inherent liability of unabsorbed radiation in deep tissue. It is possible that the increased rate of clearance subcutaneously with hyaluronidase represents marked diffusion rather than absorption.

The results of arterial and venous clamping of the common iliac vessels supports the recent view of Cullen *et al.*(7) that there is no benefit in concomitant venous ligation in acute arterial occlusion as was suggested by the early work of Makius(8) and Brooks *et al.*(9).

Summary. A study of the effect of physical factors on radiosodium clearance in dogs is reported. The average clearance rate in 25 control intramuscular injections was 59.4% activity remaining after 10 minutes. Decreases in intramuscular clearance rates were demonstrated with application of ice-salt mixtures, radiant heat and pressure cuffs. The average subcutaneous clearance rate in 10 control studies was 75.8% activity remaining in 10 minutes. This rate of clearance was increased with the addition of hyaluronidase. The clearance of radiosodium from a part does not appear as a straight line function when plotted and the calculation of per cent activity remaining per unit time is suggested as an expression for determination of clearance rates. The present study entirely supports the view of Kety that this technic is applicable to measure the effective circulation of a tissue and indicates the need for further study to quantitate the procedure for clinical application.

7. Cullen, M. L., Streppacher, L. G., Greenspan, B., Milliken, H. E., Moore, G. W., and Morris, G. C., *Surg., Gynec. and Obst.*, 1949, v89, 722.

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9. Brooks, B., Johnson, G. S., and Kirtley, J. A., Jr., *Surg., Gynec. and Obst.*, 1934, v59, 496.

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Activity of Ethyl-l-Ethanesulfonyl-4-Piperazine Hydrochloride on Traumatic Hemorrhagic Shock on the Dog. (17926)

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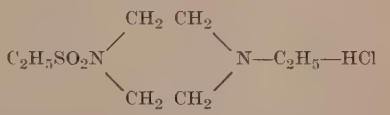
Researches, in recent years, on traumatic shock therapy, have led us to examine the effects of the following substances on shock induced in the rat by means of the drum technic (Noble and Collip), with polyvinylpyrrolidone(1), some simple aliphatic amides (2), and the protective effect exercised by a new synthetic compound, ethyl-1-ethanesulfonyl-4-piperazine (3885 R.P.)(3).



In previous experiments we tested the effect of ethyl-1-ethanesulfonyl-4-piperazine (3885 R.P.) on shock produced in the mouse, rat and guinea pig, then decided to carry out further experiments on the dog. We used the technic of Wiggers, Ingraham and Dilles (4) which produced an irreversible shock following a prolonged hemorrhagic hypotension period. The animals were treated as

* Address: Instituto Superiore di Sanita—Roma, (Italy).

1. Bovet, D., Couvoisier, S., and Ducrot, R., C.R., 1947, v224, 70

2. Bovet, D., Couvoisier, S., Ducrot, R., and Jacob, R., C.R., 1947, v224, 496.

3. Bovet, D., Couvoisier, S., Ducrot, R., and Jacob, R., C.R., 1947, 227, 1423.

described by Wiggers, under morphine or chloralose anesthesia. By extensive bleeding the blood pressure was lowered to 40 mm of kg maintained for a given time by a series of successive hemorrhages and reinjections. A total reinjection of the removed blood immediately followed the period of hypotension. The wounds were treated locally with sulfanilamide and the surviving animals were given penicillin. To minimize the seasonal or other variations which constitute the principal obstacles in this type of research, the experiments were repeated in the spring and in the autumn, and parallel experiments have been carried out in Paris and in Rome. One hundred dogs were used. The duration of hypotension was prolonged for more than the 90 minutes reported by Wiggers(4), and there resulted a larger proportion of irreversible shock in the control animals.

In the first series of experiments (I, II, III), 16 control dogs were subjected to 2 hours hypotension, with a mortality of 94%. In series IV, the control dogs exhibited a higher resistance and the period of hypotension was extended to 2½ hours.

Of the different compounds tested, polyvinylpyrrolidone and tuamine (2-amino-heptane hydrochloride), did not give any protection against severe hemorrhagic shock. Two synthetic amides, the N-N-bis-diethylamino-ethyl-ethanesulfonamide hydrochloride (3720 R.P.) and ethyl-1-ethanesulfonyl-4-piperazine hydrochloride (3885 R.P.) gave protection and the results are given below.

Compound 3885 R.P. was administered to the experimental dogs at different times, either before or after the first major hemorrhage. The most favourable results were obtained when the injection of the product immediately followed the hemorrhage. The results were less constant, probably due to rapid elimination when the compound was injected before hemorrhage, for its effectiveness decreased rapidly during the hypotensive phase.

The results are shown in Table I. There was an 88% mortality among the control animals; and 15% among the animals treated before or immediately after the hemorrhage. The period of survival was much longer in the protected animals than in control animals; 60% of the latter died within 6 hours, whereas not a single one of the treated animals died in this period.

Death from shock never occurred more than 24 hours after the hemorrhage; there was only one case of a later death in a treated animal, and this appeared to be the result of an embolism.

The untreated animals presented a clinical picture corresponding exactly with that described by Wiggers and Ingraham(3,4). Irreversible shock was constantly preceded by a marked fall of blood pressure at the end of hypotension, and by emission of bloody faeces. The treated animals practically never presented these characteristic symptoms of shock.

Discussion. On traumatic shock in the dog, we demonstrate the protective activity of certain aliphatic sulfonamides. This phenomenon was previously observed by one of us in experiments conducted with mice, rats and guinea pigs(3).

Hemorrhagic shock produced in the dog by the method of Wiggers, Ingraham and Dille, was interesting for 3 reasons: 1. The analogy between the symptoms of this type of experimental shock and the clinical shock is well established. 2. The conditions utilized in these experiments were extremely severe; with a mortality of 80 to 100% of control animals. 3. All previous attempts for protection against hemorrhagic shock have resulted in incomplete and limited protection.

Using ethyl-1-ethanesulfamide-4-piperazine hydrochloride (3885 R.P.) either before or immediately after the hemorrhage, it was possible to prevent or to retard the symptoms of shock such as cardiovascular collapse, prostration and intestinal hemorrhage, and to assure a higher percentage of survivals.

Concerning the mode of action of 3885 R.P., it has been previously reported that this compound cannot be classified in any known group of pharmacodynamic agents; it

4. Wiggers, H. C., Ingraham, R. C., and Dille, J., *Am. J. Physiol.*, 1945, v143, 126; Ingraham, R. C., and Wiggers, H. C., *Fed. Proc.*, 1945, v4, 36; Wiggers, H. C., and Ingraham, R. C., *J. Clin. Invest.*, 1946, v25, 30; Wiggers, H. C., and Ingraham, R. C., *Am. J. Physiol.*, 1946, v146, 431.

TABLE I.
Activity of Ethyl-1-Ethanesulfonyl-4-Piperazine Hydrochloride on Traumatic Hemorrhagic Shock in the Dog.

Series		Duration of hypotension, hr	Narcosis*	Controls			Experimental			
				No. of animals	Survival after re-injection, hr†	Mortality, %	No. of animals	Treatment	Survival after re-injection, hr	Mortality, %
I	Rome May, 48	2	M	5	0, 0, 3, 5½	100	5	15 min. before hemor.	12, 4 S	20
II	Paris Oct., '48 to Jan., '49	2	C	6	1-1/6 1-2/6, 2-1/2, 2-5/6, 1 S	83	6	1 min. after hemor.	10, 5 S	16
							5	20 min. after hemor.	9, 23½, 3 S	40
III	Paris May, '49	2	C	5	2, 2-1/2, 3, 5, nearly 18	100	5	20-60 min. before hemor.	20, 4 S	20
IV	Rome Oct., '49	2½ ‡	C	10	1/12, 1/8, 1/6, 1/6, 1/6, 3/4, 12, 12, 2 S	80	10	1 min. after hemor.	12, 7 S	10

* M = Morphine (5 mg/kg s.c.). C = Chloralose (100 mg/kg i.v.).
† S = Number of survivals.
‡ 5 control dogs, subjected for the same period and hypotensive-hemorrhagic shock of 2 hours, had a mortality of only 20%.

does not seem possible to explain the protective effect in shocked dog by any action in the cardiovascular system in normal animals.

The experiments do not exclude the possibility of a pharmacodynamic antagonism for one of the hypothetical toxic substances responsible for shock.

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Prothrombin Utilization during Clotting: Comparison of Results with the Two-Stage and One-Stage Methods.* (17927)

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It has been recognized for decades that prothrombin disappears from blood during clotting, but that traces of this clotting factor may persist in serum for days(1). With the development several years ago of the 2-stage method for determining prothrombin in fibrinogen-free systems(2), further studies of prothrombin and the dynamics of clotting became possible. One of us, using this method, determined the rate of prothrombin consumption during clotting of normal, hemophilic and platelet-poor bloods(3,4). Originally, the one-stage test could be used only for the study of plasma, since no separate source of fibrinogen was provided. Recently this procedure has been modified so that it can be applied to serum(5). With the modified one-stage test, serums from normal, hemophilic and platelet-poor bloods have been investigated by several workers. While the results seemed to confirm in a general way the findings with the 2-stage test, certain discrepancies were apparent.

The present study was undertaken to compare prothrombin utilization during clotting, as indicated by the 2 methods, and to determine, if possible, the basis for the discrepancies observed. Simultaneous one-stage and 2-stage determinations of prothrombin activity were performed on a series of bloods during and after clotting. Normal bloods, both human and dog, and a group of slowly clotting specimens—canine hemophilic blood, human platelet-poor plasma and normal blood clotting in silicone-treated containers—were studied.

Materials and methods. Three normal human subjects, 6 normal kennel dogs and 3 hemophilic dogs(6) were used in these experiments. Blood was collected by venipuncture in 2 dry syringes. Four ml of blood from the first syringe were mixed with 0.5 ml 3.2% sodium citrate solution and the control plasma obtained by centrifugation. Blood from the second syringe was distributed immediately into a series of 10x75 mm dry glass tubes calibrated to 2 ml (28°C). Periodically, 0.25 ml of 3.2% sodium citrate was added to each tube and the citrated serum (or plasma) obtained by centrifugation (about 3000 g for 3 minutes). For the silicone experiments, silicone-treated needles, syringes and calibrated tubes were used(7). Platelet-poor human plasma was obtained as described previously(7). One-stage and 2-stage pro-

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

† Markle Scholar in Medical Science.

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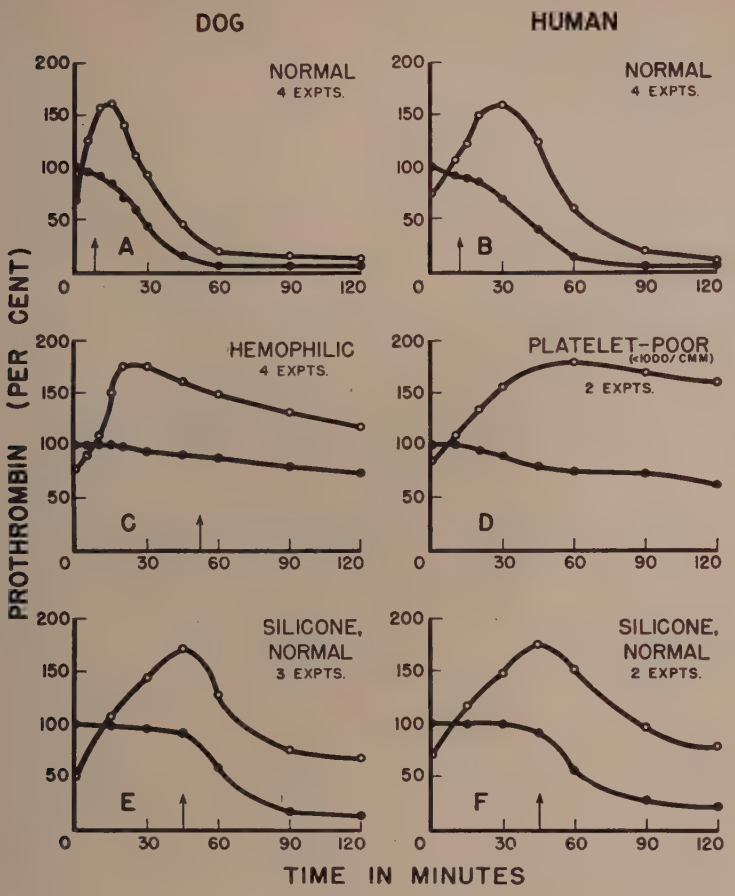

FIG. 1.

Comparison of one-stage and 2-stage prothrombin values in clotting blood. Mean one-stage determinations indicated by ○ (upper curve), mean 2-stage determinations by ● (lower curve). Arrows indicate mean clotting time (modified Lee-White method).

thrombin determinations and a test for thrombic activity were performed on all samples. Modified one-stage prothrombin times(8) were determined by mixing 0.1 ml fresh thromboplastin solution (Difco), 0.1 ml fresh normal homologous plasma treated with $BaSO_4$(9), 0.2 ml 0.02M $CaCl_2$ solution and 0.1 ml citrated test sample (37°C) in the order named. The experimental samples were tested 3½ to 5 minutes after citration, the control plasmas 40 minutes after venipuncture. Normal control dog plasma clotted between 8.2 and 8.6 seconds, human plasma between 14.6 and 15.2 seconds. Results of the one-stage test are expressed as % prothrombin activity, using plasma dilution curves(9) with the 100% values obtained from the control plasmas. For values higher than 100%, the sample was diluted to half-strength with $BaSO_4$-treated plasma before testing. Percentile yields were then doubled. Serum thrombic activity was determined

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simultaneously with the one-stage prothrombin tests by adding 0.1 ml citrated serum to 0.1 ml homologous $BaSO_4$-treated plasma and 0.3 ml 0.9% NaCl (37°C). Two-stage prothrombin determinations(2) were performed with added beef serum(10). Normal dog plasma contained 375 to 425 prothrombin units per ml, normal human plasma 275 to 325 units. Results are expressed as % of the appropriate control plasma.

Results. In Fig. 1 the results obtained during the first 2 hours of these experiments are summarized. As measured by the 2-stage method, the prothrombin content of the serum diminished progressively, but at varying rates in the different types of experiments. With the one-stage method, a period of hyperactivity in which the values exceeded 100% was noted soon after the blood was drawn. The serum prothrombin clotting times reached a minimum of about 6.0 seconds for the dog serum, and about 10.5 seconds for the human serum. The hyperactive phase was greatly protracted in the hemophilic and platelet-poor samples (Fig. 1, C and D). Data from 6 experiments on canine hemophilic blood, not included in the chart, showed that the period of hyperactivity lasted for 3 to 4 hours. In 2 experiments with platelet-poor human plasmas, this hyperactive period lasted 6 to 7 hours.

Little or no thrombin was found in the citrated serum samples. With the dog serum, no clots were observed in 4 hours. With the human serum, the thrombin clotting times varied greatly; a few samples clotted in 5 minutes, but most were not clotted in 4 hours.

With the original 2-stage prothrombin procedure(2), the incubation time required for maximum thrombin activity was regularly shorter for the serum samples than for the plasma samples. Optimal incubation times were determined carefully in several experiments, using Ac globulin-poor beef lung (Mg $(OH)_2$ treated) as thromboplastin. The optimal activation time for the human plasma was 5 minutes, compared to 3 minutes for serum of approximately the same prothrombin unitage. Although the rate of thrombin formation was more than 1½ times as fast in serum, prothrombin values were never higher than in the control plasma. Similar results were observed in canine experiments.

Discussion. During the early stages of these experiments, the one- and 2-stage procedures gave divergent results. The slower the clotting, the more prolonged was the period of divergence. After the peak of prothrombin activity observed by the one-stage method, the 2 curves tended to converge. However, the one-stage results remained consistently higher than the 2-stage values. The high serum activity observed by the one-stage test does not appear to be due to residual thrombin(11), but rather to the elaboration of an accelerator of prothrombin conversion during clotting(12,13,14). An attempt was made to obtain better agreement between the results with the 2 methods by recalculating the one-stage values on the descending portions of the curves. The highest one-stage result in % was made equivalent to the corresponding 2-stage value in %. The subsequent one-stage values then were adjusted proportionately. With this recalculation the one-stage values became almost identical with the 2-stage values. This suggests that if the one-stage procedure were modified further by addition of the serum accelerator factor, the discrepancies in the serum values obtained by the 2 methods might disappear. It will be noted that the peak of hyperactivity of serum in the hemophilic and platelet-poor samples (Fig. 1, C and D) is approximately the same as in the corresponding normal bloods. The hyperactivity of hemophilic serum was evident in the data presented previously by Quick(5). It was not recognized at the time however, and in retrospect, it is easy to see how this was interpreted as evidence for a "quantitative lack of conversion" of prothrombin dur-

10. Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.*, 1949, v19, 471.

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12. Owren, P. A., *The Coagulation of Blood,* J. C. Gundersen, Oslo, 1947.

13. Ware, A. G., and Seegers, W. H., *Am. J. Physiol.,* 1948, v152, 567.

14. de Vries, A., Alexander, B., and Goldstein, R., *Blood,* 1949, v4, 247.

ing the clotting of hemophilic blood.

It will be noted in Fig. 1 that the initial one-stage prothrombin values were lower than those obtained with the control plasma. Thus, in the normal human experiments (Fig. 1 B), the prothrombin time of the control plasma was 14.8 seconds (100%), while in the experimental series the plasma prothrombin time was 16.2 seconds (74%). The chief difference between these 2 plasmas was the age at the time of testing. The one-stage plasma determination in the experimental series was made within 5 minutes after venipuncture, while in the control plasma the test was not made until 40 minutes after venipuncture. Other studies have shown that during the first 30 to 45 minutes after blood is collected, the prothrombin time of citrated plasma gradually shortens. These findings are in accord with those of Owren(12), and indicate the importance of the time elapsing between collection of blood and performance of the one-stage test. While prothrombin times longer than 11 or 12 seconds on normal human plasma by the usual method are generally attributed to a relatively inactive thromboplastin, they may actually be due to the prompt testing of freshly collected plasma. Further studies are needed to determine whether the changes in plasma prothrombin time immediately after venipuncture are due to the same factor(s) that causes hyperactivity of serum in the one-stage test.

Summary. 1. A comparison was made of the changes in prothrombin during clotting, as indicated by the one- and 2-stage methods.

2. By the 2-stage method, progressive disappearance of prothrombin from serum was observed. Prothrombin utilization was slower in human blood than in dog blood, and was delayed greatly in canine hemophilic blood, platelet-poor human plasma, and in blood clotting in silicone-treated glassware.

3. By the one-stage method, an initial period of hypoactivity in the plasma was followed by a hyperactive phase in the serum. At the peak of hyperactivity, "prothrombin" values were about 180% of the control plasma. In slowly clotting bloods, the hyperactive phase developed less rapidly and persisted for a longer period than in normal blood. The abnormally high serum prothrombin values obtained by the one-stage test appear to be due to the evolution and persistence of the recently recognized serum factor which accelerates thrombin formation. Apparently this factor does not influence the 2-stage serum prothrombin values.

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A Diabetes Insipidus-like Condition Produced in Dogs by a Potassium Deficient Diet. (17928)

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(Introduced by D. T. Smith)

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It is well known that administration of desoxycorticosterone acetate, an indirect means of reducing the serum potassium level, results in the production of a syndrome in dogs of polydipsia and polyuria resembling diabetes insipidus(1-3). To our knowledge the effect of direct dietary withdrawal of potassium on the fluid exchange has not been reported.

Twelve dogs housed in metabolism cages were placed on basic diets* containing approximately 0.01% potassium. The details

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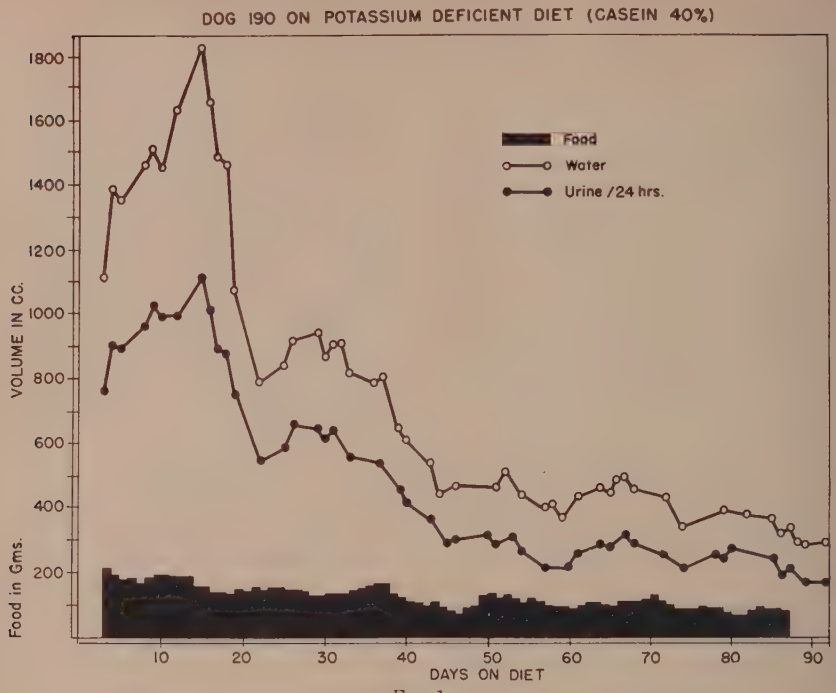

FIG. 1.

The fluid exchange (water intake and urine output in ml) of a dog on the 40% casein diet is expressed as a moving mean plotted against the time in days. The food is expressed as the actual value indicated on a given day.

of these diets* were given in a previous communication(4). Five additional dogs served as controls, 2 receiving potassium at a level of 0.24% as potassium chloride introduced into the salt mixture. The other 3 received potassium at approximately the same level but supplied in the form of brewer's yeast at a level of 10% replacing an equivalent amount of sucrose. The yeast also supplied any possible missing B-complex factors of the diet. As far as the fluid exchange was concerned there was no difference in the two control diets but the general well being was far superior in the yeast-fed dogs. Those receiving the yeast always lived throughout the experimental period and remained in excellent condition whereas the controls receiving the 8 synthetic B-complex factors plus potassium chloride usually were in poor condition and died during the experimental period, the average longevity of 6† dogs being 43 weeks. Two of the yeast control dogs have been allowed to continue on the experimental diet for periods of 5 and 6 years respectively. They are both living and in excellent condition.

When the 24-hour urine volumes (15 - 520 cc) of potassium depleted dogs after various periods on the diet were compared with those

* Composition of basic diets (1) high protein; casein 40%, sucrose 44, cotton seed oil 10; cod liver oil 2, salt mixture 4% (2) low protein: casein 20, sucrose 64, cotton seed oil 10, cod liver oil 2, salt mixture 4%. The fat soluble vitamins A, D, E, and K dissolved in oil; the B-complex factors, thiamin, riboflavin pyridoxine, nicotinic acid, pantothenic acid, para-aminobenzoic acid, inositol and choline dissolved in water were fed individually to each dog.

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† Two of these dogs were used in this investigation and 4 in a previous study.

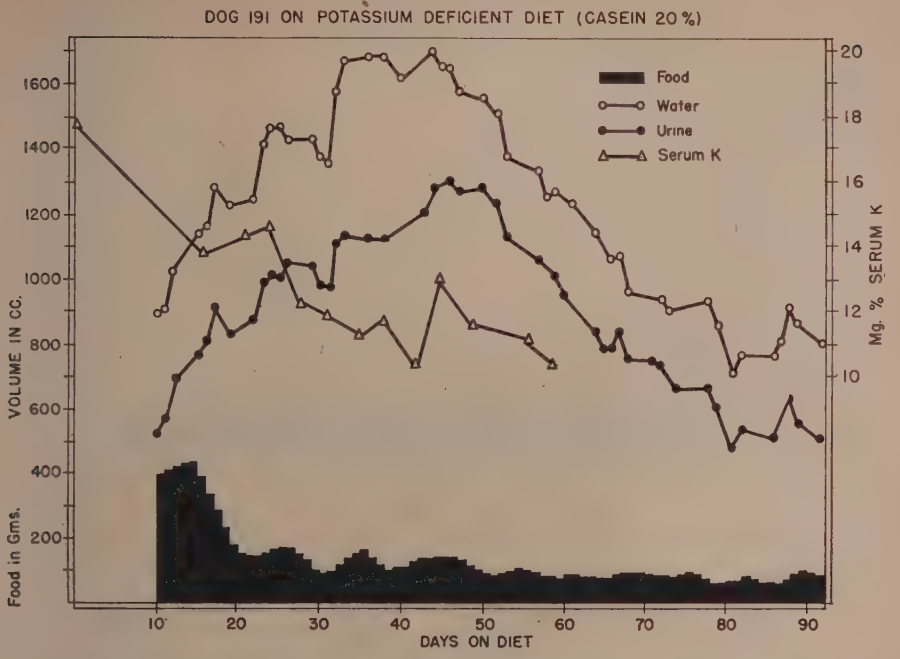

FIG. 2.

The fluid exchange of a dog on the 20% casein diet expressed as a moving mean against time in days. The food and serum potassium values are expressed as the actual value on a given day.

(10-1665) of controls receiving potassium, the former were usually much greater, but because of the tremendous variation in output the differences were often not statistically significant. It seemed likely, however, that the urine excretion was following a pattern that might be consistent if discernible. We, therefore, used a moving mean‡ to smooth out the curve and thus show the pattern. The first diet used in these experiments contained 40% casein, which was later reduced to 20% at the expense of the carbohydrate (sugar) component. On both diets there was a sharp rise in volume beginning often within 24 hours after the animal was placed on the deficient diet. After the peak was reached the volume of output decreased just as rapidly as it had risen. Eleven of the dogs were studied in great detail, 3 on the high protein (40% casein) diet, 3 on the normal protein (20% casein) diet and 5 on the control diets. This is not a large series but the consistency of the results is rather impressive. The high (40%) casein diet showed the expected sharp rise with a peak at 13, 16 and 31 days respectively in these 3 dogs (Fig. 1). The normal protein of 20% casein resulted in a shift of the peak to the right at 37, 46 and 50 days respectively (Fig. 2). The control dogs showed a fairly smooth curve with a daily urine output averaging less than 300 cc while one potassium deficient dog went as high as 1710 cc (Fig. 3).

The urine volume excreted by the different dogs is about what would be expected if the pattern described is followed consistently. The

‡ The moving mean as applied in this study consisted of the averaging of the first 5 values for urine output, etc. The mean obtained from the sum of these figures would then correspond with the 3rd figure. The 1st figure is then dropped, the 6th added and a mean obtained which in turn corresponds with the 4th figure and so on through the data. In this way (using 5 figures) 4 points are sacrificed, two at the beginning and 2 at the end of the curve but it is smoother and reflects the trend more clearly.

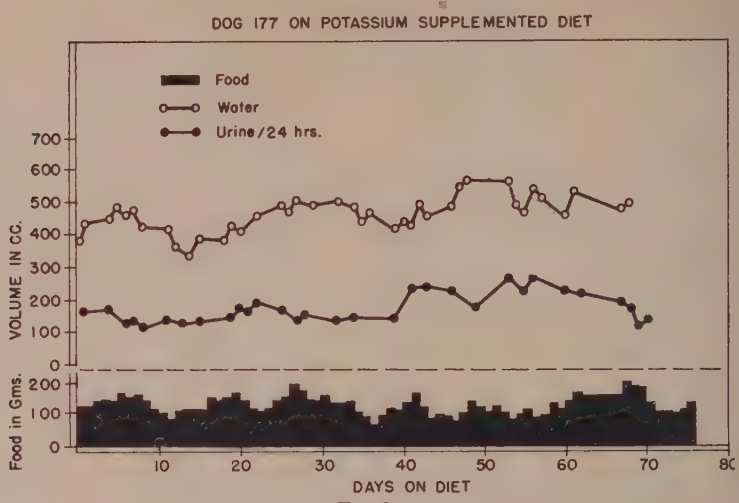

FIG. 3.

The fluid exchange of a potassium control dog represented as a moving mean against time expressed in days. Food is expressed as the actual value observed on a given day.

performance of Dog 6 is interesting in this respect. Eleven 24-hour urine volumes taken after 9 weeks on the deficient diet averaged 327 cc. The dog later became paralyzed, was placed on the potassium control diet for 6 days during which time the following consecutive daily volumes were recorded, 120, 535, 770 and 900 cc per day. The dog was then switched back to the potassium deficient diet and the next eleven consecutive volumes recorded averaged 1049 cc per day.

The urine excretion of another dog recorded during the 24 hours preceding a paralytic attack was 960 cc. At the time of the attack, May 11, the dog was treated with biotin (400 mg every hour for 9 hours), then maintained on biotin (800 mg per day given subcutaneously in 2 - 400 mg doses) for a period of 10 days. The next 5 days on which urine was measured (May 14 - 18 inclusive) the volumes excreted were 555, 590, 645, 570 and 500. Then the volume began to increase and rose to 1105 on May 21st. During that day the dog experienced another paralytic attack which was treated with potassium chloride (5 g orally). Urine volumes recorded from May 23 - June 4 were as follows: 970, 1320, 1740, 1520, 1490, 840, 1725, 1235, and 1140 cc/24 hr.

In our earlier studies it was observed that the dogs usually became paralyzed well after the tendency to polydipsia and polyuria had spent itself and the curve of urine output had reached a plateau within or very near the normal level. Biotin was found to reverse the paralytic process though less satisfactorily than potassium. The dog cannot be maintained on biotin free from paralytic attacks for more than 2 weeks. To determine the effect of biotin on the fluid exchange, 4 dogs were treated with 800 mg per day subcutaneously for 2 weeks. In each case the average urine volume dropped 51.7, 39, 33 and 69% respectively for the first week but for the second week the average was as high or higher than the predosing level. In the 2 control dogs treated with biotin there was no consistent change.

As the urine output and water consumption increases the food consumption and the level of serum potassium decreases. The serum potassium falls gradually from a normal value of 17 - 20 mg % to 10 - 12 mg % where it remains for the most part until the dog becomes paralyzed. The paralysis in most cases starts a new cycle. The period of depletion is so long that no dog has been followed metabolically for the entire time but

isolated instances indicate that the polydipsia and polyuria start up again and are unchecked by the potassium therapy which so effectively controls the paralytic episodes. This was also true of the diabetes insipidus and paralysis produced in dogs receiving desoxycorticosterone acetate(3).

The pH of the urine remained the same in a given dog, on all diets and during the successive changes in fluid exchange varying from 8.2 - 9.2.§ These values were obtained daily on 3 animals for 10 days on the yeast control period—then for a similar period after being placed on the potassium deficient diet—and later once a week.

Discussion. An unusual pattern of polydipsia and polyuria has been observed in dogs following the withdrawal of potassium from a synthetic diet. The onset of this increased fluid exchange begins rather precipitously after placing the dog on the deficient diet. The peak of the water intake and urine output occurs usually in 3 - 7 weeks while a gradually progressive dehydration takes place during the entire period of potassium depletion. The polydipsia and polyuria seems to bear no relation to the blood potassium level. Nor does this phenomenon parallel the usual clinical symptoms or signs of potassium deficiency as previously described(4). The acute spasticity and progressive paralysis observed usually occur long after this heightened fluid exchange has subsided. No adequate explanation for this abnormal fluid exchange has yet been found. It could be related to an electrolyte readjustment scheme initiated by the organism for the purpose of preserving normal osmotic relationships(5). The transient nature of this phenomenon during the continued course of potassium depletion suggests the possibility of mediation through the adrenal cortex(6,7). Should the process depend upon a hormone of the cortex the cessation could be explained by the depletion of some essential component necessary for the elaboration of this hormone. More data are needed to test these hypotheses and experiments are now in progress to this end.

§ We are grateful to Dr. Hilda Pope for having these determinations made in her laboratory.

Conclusions. Dogs depleted of potassium show a marked increase in fluid exchange. The increase begins usually within 24 hours after being placed on the potassium deficient diet provided the dog is healthy at the start of the experiment, a condition we have assured in our dogs by placing them on a yeast control diet for 3 weeks preexperimental period. A peak in the curve is reached after 3 - 7 weeks and then there is a gradual decline back to nearly normal values. Factors responsible for this phenomenon are still undetermined.

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The brewer's yeast was supplied by Standard Brands, Inc., through the courtesy of Dr. Charles N. Frey.

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Lymphocytic Response of Normal Individuals to Transient Hyper- and Hypoglycemia. (17929)

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(Introduced by Co Tui)

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Prompt secretion from the adrenal cortex is elicited by a great variety of stress phenomena(1). The increase in the level of 11-oxycorticosteroids thus produced, causes a reduction in the absolute lymphocyte count, which correlates well with the reduction in adrenal cortex cholesterol level(2). As this latter level is a good index of adrenal cortical activity(3) the lymphocyte response can be used as an indicator for the same purpose. Lymphocytopenia is produced in normal animals(4) and man(5) by the oral administration of glucose, but not in adrenalectomized animals or in Addisonian patients(6). Similarly, hyperglycemia after oral or intravenous glucose in dogs, causes eosinopenia(7), indicating increased adrenal cortical activity. Insulin, administered in sufficient dosage to produce shock, also causes lymphocytopenia both in animals(8) and man(9). However, recent observations showed that, in the rat, adrenal cholesterol increased following oral glucose, and decreased after insulin hypoglycemia(10). Since these results partially contradict those quoted previously, a study in the human of the effect on the absolute lymphocyte response of hyperglycemia and moderate hypoglycemia was attempted.

Materials and methods. Fasting capillary blood samples for glucose were obtained from 6 non-diabetic individuals of average weight and nutritional status. After receiving 25 g of 50% glucose solution intravenously, capillary blood for glucose determinations was collected at 30, 60, 120, and 180 minutes, and simultaneously, samples for the total white blood cell count and for smears for differential counts were taken. Five individuals of this group, and 3 additional persons of similar status, received 0.075 unit of regular insulin per kilo of body weight intravenously. Capillary blood was withdrawn immediately before injection of insulin, and after 20, 30, 45, 60, 90, 120, and 150 minutes, for glucose determination, for total white cell count, and for smears for differential counts. The blood sugar was determined by the Folin micro-method(11). The percentage of lymphocytes was determined by taking the average of three differential counts of 300 cells each.

Results. The blood glucose level at ½ hour was within normal limits for all individuals, and then declined to fasting values at the end of 2 hours. The absolute lymphocyte count decreased in all cases after glucose administration. In 4 patients the lowest count was reached within ½ to 1 hour. In one individual, the lowest level was not reached before 2 hours, and in another person, there was a marked drop at 1 hour, and a second peak drop at 3 hours. The mean rise of blood sugar level compared with the mean decline of absolute lymphocyte count is seen in Fig. 1.

The lowest blood sugar level, after insulin,

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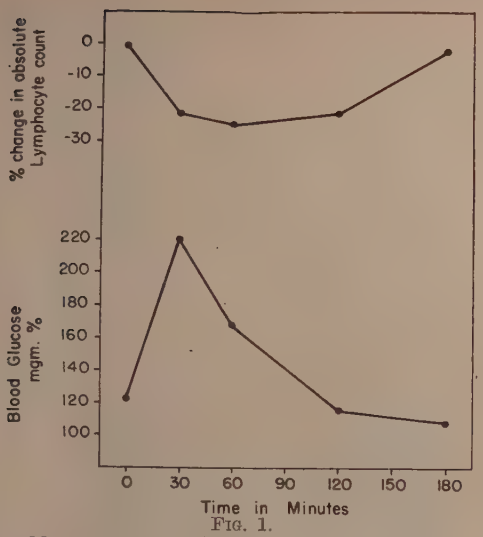

FIG. 1.

Mean percentage change of absolute lymphocyte count in 6 patients following intravenous administration of 25 g of glucose as 50% solution.

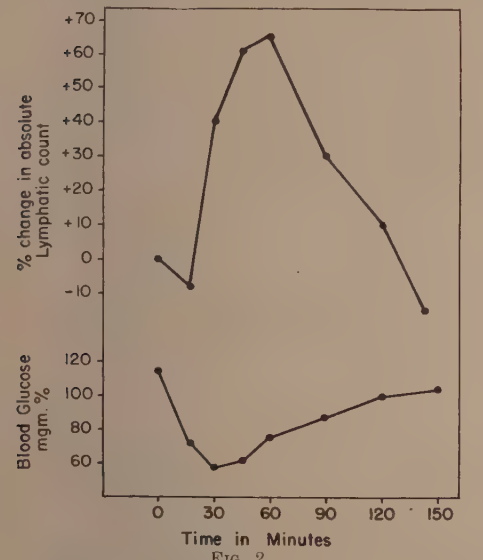

FIG. 2.

Mean percentage change of absolute lymphocyte count in 8 patients following intravenous administration of 0.075 unit of regular insulin per kilo of body weight.

was reached within 20 to 30 minutes, whence it rose gradually. Seven out of 8 individuals showed a marked absolute lymphocytosis, which reached its peak at 45 or 60 minutes. In one subject, the rise was delayed until 120 minutes. In 4 persons the absolute lymphocyte count started to rise immediately, whereas in the other 4 cases, there was an initial small drop followed by a rise. None of the patients showed symptoms of insulin shock. The mean values of blood sugar levels and per cent changes in absolute lymphocyte counts are shown in Fig. 2.

Discussion. From the data it appears that, in the normal individual, administration of glucose causes lymphocytopenia, whereas moderate insulin hypoglycemia causes lymphocytosis. It may then be assumed that hyperglycemia stimulates, whereas moderate insulin hypoglycemia inhibits, adrenal cortical secretion. Our findings do not coincide with those of other investigators(8,9) who reported lymphocytopenia after administration of insulin. This apparent discrepancy may be attributed to the fact that the dosage used by them was sufficient to produce shock, whereas with the dosage employed by us, none of the individuals showed symptoms of hypoglycemia, thus avoiding the stress factor. The role of the adrenal cortex in the mechanism for the regulation of the blood sugar level has not as yet been clarified. It is usually considered to oppose the action of insulin, and to raise the blood sugar level. Our observations, however, suggest a response of the adrenal cortex both to hyper- and hypoglycemia, within physiologic limits, which helps to maintain homeostasis. A similar view has been expressed by others(12,13) on the basis of animal experiments. In support of this hypothesis, are the observations that cortical extracts retard the fall of glycogen in perfused livers(14), and that there is, in experimental animals, an inhibition of adrenalin hyperglycemia by cortin(15), thus indicating an anti-glycogenolytic activity for the cortical hormone. In addition, when glucose and 11-oxysteroids are administered to

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the adrenalectomized animal, more liver glycogen is deposited than when either is administered alone(16).

These findings suggest that the stimulation of secretion of adrenal cortical hormone by exogenous glucose, in the normal individual, allows more of the administered glucose to be taken up by the liver and stored as glycogen, thus enabling the blood sugar level to return rapidly to normal. On the other hand, the inhibition of the adrenal cortex caused by hypoglycemia should tend to restore the blood sugar level to normal, probably by allowing glycogenolysis to take place.

There is an alternative explanation of the lymphocytosis after insulin, based on the finding that initially epinephrine administration causes lymphocytosis(17). This explanation is, however, partially negated by the fact that adrenodemedullated animals exhibit a normal insulin tolerance curve(18), and it is therefore unnecessary to assume that moderate hypoglycemia elicits epinephrine production.

Summary. The intravenous administration of 25 g of glucose in 50% solution was accompanied by a significant lymphocytopenia which reached its lowest level at ½ to 1 hour in 5 normal subjects and at 2 hours in the sixth. The intravenous administration of 0.075 unit of regular insulin per kilo of body weight evoked a significant absolute lymphocytosis which reached its peak at 45 to 60 minutes in 7 normal persons, and at 120 minutes in the eighth. The lymphocytopenia after glucose administration is inferred to be a result of adrenocortical stimulation caused by the change in the blood sugar level. The absolute lymphocytosis after insulin is similarly assumed to result from adrenocortical inhibition due to the moderate hypoglycemia. The discrepancy between our findings and those of others, who noted a decrease of lymphocytes following insulin, is explained by the small amounts of insulin used by us, thus avoiding shock and concomitant adrenocortical stimulation. This study suggests that the response of the adrenal cortex to hyper- and hypoglycemia serves to maintain homeostasis.

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The authors wish to express their appreciation for the technical assistance of Miss M. Gilady, Miss A. Spiegelman, and Mrs. E. S. Lazarus.

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A Small Bore Polythene Shunt to Prevent Mechanical Shock after Prolonged Cross-Clamping Thoracic Aorta.* (17930)

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Prolonged cross-clamping of the patent thoracic aorta distal to the left subclavian artery is uniformly followed by severe neurological and visceral damage(1,2,3,4,5). Initially, the injury is not dependent upon cardiac failure, but upon the effect of diminished circulation to those susceptible structures distal to the cross-clamped area. Later effects are consistent with a progressive reduction in an effective circulating blood volume as this fluid pools in the anoxic, hypotensive region(1). It is therefore necessary

* This work was supported by grants from the United States Public Health Service, the Graduate School of the University of Minnesota, and the Robert Ferguson Surgical Research Fund.

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to deal satisfactorily with the problem of hypotension and tissue anoxia before extensive, time consuming surgery, on the thoracic aorta becomes feasible.

Experimentally, Carrel diverted the flow through a paraffined glass tube temporarily placed within the aorta or by a tube from the left ventricle to the descending aorta(3). Gross and Hufnagel, by refrigeration of the backs of dogs, lowered the metabolic rate of the tissues, and thus avoided neurologic damage in three dogs in which the aorta was clamped 45 minutes(4). Clinically, Crafoord has limited his operating time on the clamped aorta to about 30 minutes(5). Potts, Smith, and Gibson have recommended the use of a partially occluding clamp on the aorta(6). While all methods mentioned are appropriate solutions to a particular problem, none present the surgeon with an unencumbered field in which he can carry out prolonged procedures on the circumference of a bloodless thoracic aorta.

Experiments. Thirty-one mongrel dogs weighing 6-20 kg were anesthetized with 30-35 mg I.V. sodium pentobarbital, intubated and connected with a standard anesthesia machine delivering a high concentration of O_2. In an aseptic field the left chest was opened through the bed of the fourth rib supplemented when necessary by cutting the third and fifth ribs. The aortic arch, descending aorta and subclavian artery were widely dissected with division of the superior 2 or 3 pairs of intercostal arteries and the ligamentum arteriosum. A silicone coated polythene tube 12-15 cm long, with an inside diameter of .062″ for dogs under 10 kg or .085″ for dogs over 10 kg was then fashioned into a suitable form by molding in hot water. In some instances a flange of polythene had previously been affixed to the proximal end prior to use. The proximal funneled or flanged end of the tube was then inserted into the clamped subclavian artery, the aorta was then clamped and the turned up distal end of the tube passed between 2 sutures through a puncture wound into the distal aorta. Upon removal of the proximal subclavian clamp blood swelled the distal aorta. In 5 dogs continuous mean pressure recordings were obtained in the carotid and femoral arteries. The femoral pressures ordinarily dropped to levels of 20 to 30 mm Hg upon cross-clamping. The carotid pressure rose to 180-200 mm Hg. Upon opening the shunt the femoral pressure rose to 50-70 mm Hg and the carotid pressure fell to about 160-180 mm Hg. Over a period of 2 to 5½ hours the carotid pressure gradually fell to near normal levels of 120-140 mm Hg. The femoral pressure fell to 40-50 mm Hg.

In 26 dogs segments of aorta were removed and autogenous grafts implanted or "notches" made in the aorta and the remnant banded to produce a coarcted area(7). In no instance did the siliconed shunt thrombose. No supportive therapy in form of blood transfusions or stimulants was administered.

Results. In the control group weighing 8-17 kg, in which the aorta was clamped more than 30 minutes, 10 of 13 dogs had permanent spastic paralysis or died within 12 hours of mechanical shock.

In the "shunted" group weighing 6-20 kg, all clamped more than one hour, one 20 kg dog with a .085″ shunt in place died immediately with uncontrollable tachycardia, ventricular fibrillation and cardiac failure after slowly removing the aortic clamps.

This remains an unexplained phenomenon since it was a solitary occurrence among all the experiments performed. In the remaining 14 animals there were no instances of mechanical shock, or spastic paralysis. Four dogs showed slight stiffness of the hind legs which lasted less than 72 hours.

Discussion. The deleterious effect of cross-clamping of the thoracic aorta may be due to one or many factors. Shorr *et al.* demonstrated that vasodepressor substance is released by skeletal muscle and liver cells in response to anoxia(8). Fine *et al.* have pre-

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vented the irreversibility of hemorrhagic shock by ingenious cross perfusion of the liver(9). It is not our purpose to attempt to evaluate the factors involved in the development of spastic paralysis or irreversible shock, but rather to make another practical use of the fact that tissue damage due to arterial hypotension is related to the product of the degree of hypotension and the time that it persists.

In this instance one can by an inexpensive, readily procurable tube, which can be fashioned into the desired form at the operating table, make a temporary channel to divert blood into a temporarily hypotensive and anoxic area. Although such a small shunt does not return the deprived circulation to normal it enables one to bargain for the time necessary to accomplish a definitive procedure.

The use of similar devices to provide for diversion around smaller essential vessels, such as the coeliac, superior mesenteric or common femoral or hepatic artery, which are undergoing reconstruction might be indicated.

Conclusions. A small polythene shunt used to divert a portion of the blood around the cross-clamped thoracic aorta will temporarily prevent mechanical shock in dogs.

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Use of a Glass Edge in Thin Sectioning for Electron Microscopy.* (17931)

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The use of a steel microtome knife in cutting sections for electron microscopy has certain disadvantages, especially in the achievement and maintenance of a sufficiently sharp edge, which involve a considerable expenditure of time. The search for a knife material having homogeneity and hardness without excessive brittleness led to the development of a glass cutting edge. With it, we have been able to cut thin sections more consistently, easily, and rapidly than with steel microtome knives. Since the glass "knives" are obtained simply by breaking them from a strip of glass, the tedious and uncertain sharpening procedures necessary with steel knives are eliminated. Inspection with the light microscope reveals a smoother and, we believe, sharper edge. Fewer grooves or scratches are found on the face of a block of tissue after it has been cut with the glass edge. The glass knives are also quite inexpensive. The knives are made by breaking a strip 1½″ wide and about 12″ long from a sheet of plate glass approximately 3/8″ thick. Since the edges thus produced will form clearance facets, this fracture should be as smooth and straight as possible. A series of straight parallel scorings at 45° to the long axis are then made on each strip, 1″ apart, and on the opposite surface from the first scoring. The parallelograms thus outlined are broken off, producing a set of glass blocks, each of which has two cutting edges 3/8″ long, formed by faces meeting at a 45° angle (Fig. 1,A). Any competent glass cutter with standard equipment can produce these knives from stock plate glass for something less than 25 cents each. Straighter edges, however, can be more regularly secured with a diamond-point scriber having constant spring tension (designed by Robert C. Jackson of the Department of Biology Machine Shop), utilizing a controllable pressure device, such as an arbor press, to break off the knives.

The knife holder, as shown in Fig. 1, was

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† Postdoctorate Research Fellow of the National Cancer Institute, National Institutes of Health.

‡ Visiting Fellow in Biology, on leave from the Department of Anatomy, University of Minnesota.

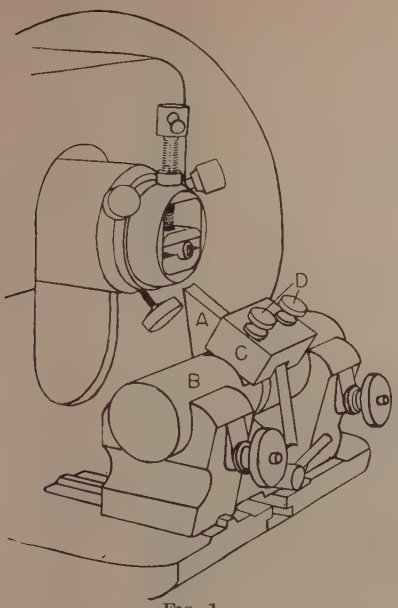

FIG. 1.

constructed to fit the knife clamp assembly of the Spencer microtome, but it can easily be modified to fit other microtomes. It seems desirable to retain the rotatable feature so that any desired clearance angle may be obtained. The holder consists simply of a steel rod (B) of the proper diameter to replace the clamps in the knife clamp assembly. At its midpoint the holder presents a slot, milled at right angles to the long axis of the rod, about 7/8″ deep and just wide enough to accommodate the thickness of the glass used. A yoke (C) with a shallow slot is held in alignment with the slot in the rod by a pair of thumb screws (D). The glass knife is placed in the slot with the facet produced by the first fracture facing the object holder. After it is clamped by screwing the yoke in position, the clearance angle is adjusted by rotating the holder in the knife clamp assembly of the microtome. A clearance angle of about 10° has been found suitable for sectioning material embedded in polymerized butyl methacrylate(1,2). If desired, a water trough to float sections off the edge of the knife(3) may be made from a piece of light cardboard, waterproofed and attached with paraffin or common plastic cement.

The second fracture, *i.e.* the one produced when a knife is broken from the glass strip, will rarely fall on a plane at right angles to the original surface of the glass. As a result, the cutting edge will be either tilted a little from the horizontal, or curved. In either case, the edge seems to cut fully as well as, if not better than, the occasional straight and horizontal edges, possibly because of shearing action during the cutting stroke. The orientation of the leading and trailing edges of the tissue block with respect to the knife edge appears to be unimportant, but when the block edges are parallel and aligned with the knife edge, ribbons of sections may be formed.

Some results obtained with central and peripheral nerve tissue are illustrated in Figs. 2 and 3. The tissue was fixed in 10% formalin, followed by 2% osmic acid, then dehydrated and embedded in n-butyl methacrylate(1,2). Before study in the electron microscope, the methacrylate was removed from some sections with toluene and was allowed to remain in others.

The microtome feed may be adjusted for thin sectioning by the wedge method suggested by Baker and Pease(4) or by the thermal expansion technic of Newman, Borysko and Swerdlow(1,2). This latter procedure may be simplified by merely cooling the tissue block (clamped directly in the jaws of the object holder) with a stream of CO_2 from a tank just prior to cutting. With the microtome feed disengaged, the slow expansion of the tissue block will permit a number of thin sections to be cut before recooling is necessary. This avoids torque on the horizontal ways of the microtome due to the coupling of a high-pressure hose. Cooling also facilitates the cutting of softer blocks.

The useful life of the glass edge compares favorably with that of a freshly-sharpened

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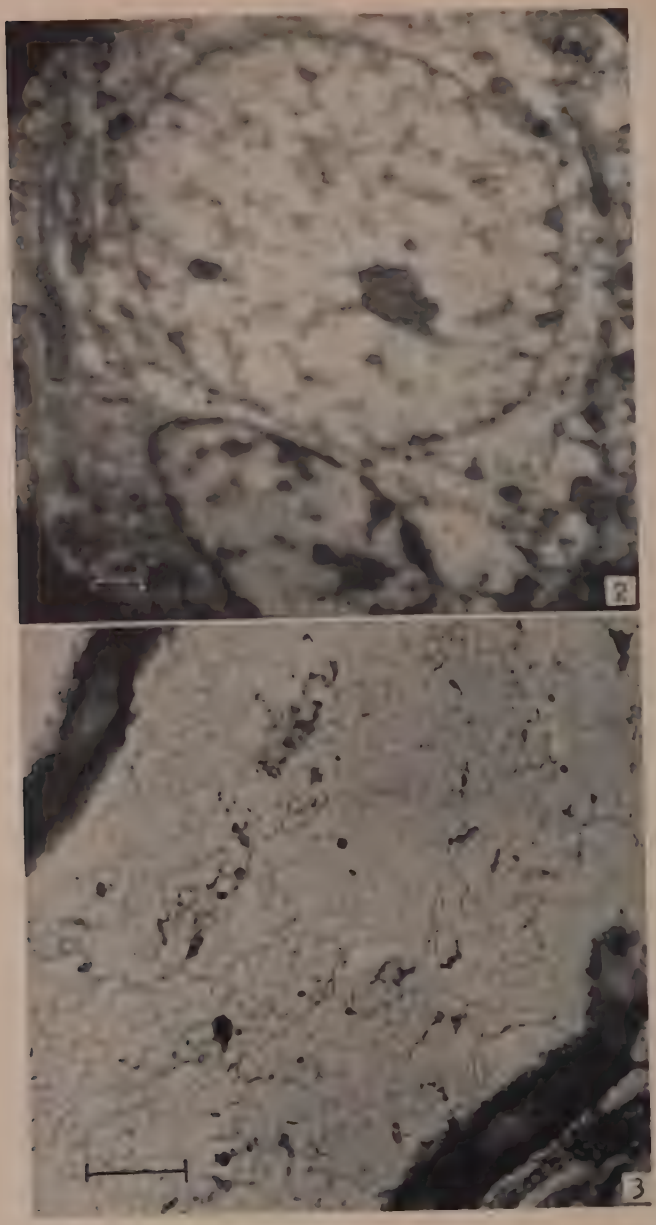

FIG. 2. Section of cerebral cortex of rat, with embedding medium left in. × 7,000.

FIG. 3. Longitudinal section of axone in sciatic nerve of rat Embedding medium removed. × 14,000.

steel edge. When a glass edge is dulled it is discarded. It might be possible to obtain more durable edges from industrial diamonds or artificial sapphires when improved techniques

for polishing cutting facets are developed.

The authors wish to acknowledge with thanks the assistance of Mr. William Strovink of the Biology Machine Shop in developing the knife holder.

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Absorption and Distribution of Vitamin A in X-Irradiated Rats.* (17932)

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The gastro-intestinal mucosa is one of the tissues most sensitive to ionizing irradiation. Various studies have indicated physiologic alterations in gastro-intestinal function following irradiation with impaired absorption of certain foods. In the comprehensive review of Warren and associates(1), several experiments are cited which indicate impaired absorption of sugars and fat. Studies on absorption in radiated rats indicated a diminished absorption of glucose at 24 and 40 hours following irradiation(2). This has been recently confirmed by Barron(3) in rats at 2 and 4 hours post-radiation. Impaired and altered fat absorption has also been described in human leukemia following irradiation(4). Studies on dogs receiving a moderately large dose of x-ray to a single loop of the bowel showed no fat in the lymphatics of the radiated loop after a test meal(5). Certain indirect evidence, however, suggested that the absorption defect following whole body irradiation in the mid-lethal range cannot be as large as those earlier experiments suggested. Dogs show little weight loss following irradiation with mid-lethal doses; and such weight losses as are observed can be nearly accounted for by anorexia which is frequently seen(6). It would seem reasonable that if absorption were more than minimally impaired, the difference in weight between the irradiated animals and pair fed controls would be much larger.

The findings in the present studies indicated that, after the first day, post-radiation absorption of vitamin A alcohol is essentially normal or increased; however, there is an abnormality in the tissue distribution.

Methods. In these studies young female rats of the Wistar strain were used. The rats were fed a standard Purina Fox Chow mixture ground into a fine granular form. The irradiated rats received 625 r whole body radiation which is an LD_{50} in our laboratory. Rats were studied at intervals from one hour post-radiation up to 7 days post-radiation. The test dose of vitamin A alcohol was administered to irradiated and control rats by stomach tube from a tuberculin syringe. During the test period, the animals were allowed water and food *ad libitum*. The animals were anesthetized with chloroform and exsanguinated from the vena cava at either 6 or 10 hours. At autopsy, the gastro-intestinal

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TABLE I.
Percentage Dose of Vitamin A Recovered 6 Hours After Administration.

Time post-radiation		Intestine	Liver	Carcass	Total recovery
3 days	R*	51.4	10.1	24.5	86.0
	C	60.7	13.6	18.6	92.9
3 "	R	29.0	8.6	16.2	53.8
	C	58.0	11.4	5.5	74.9
3 "	R	23.5	4.1	34.0	61.6
	C	62.5	7.8	4.6	75.1
3 "	R	24.8	3.3	12.0	40.1
	C	55.5	8.0	2.4	65.9
3 "	R	78.3	3.8	2.7	84.3
	C	80.2	5.4	1.8	87.4
3 "	R	32.0			
	C	65.0			

* R = Radiated. C = Controls.

tract was removed in 2 segments. Ties were placed at the pylorus in order to avoid loss of either gastric or intestinal contents. Recoveries of vitamin A were carried out on the stomach, small and large intestine, liver, and remaining carcass of each rat. The adrenal glands were pooled for each group of control and radiated animals. Both the esophagus and trachea were removed and analyzed with the stomach. The tissues were frozen and stored until used. The tissues were homogenized in a Waring Blender in alcohol in the presence of hydroquinone, and then were analyzed for vitamin A by the Clausen and McCoord modification of the Carr-Price reaction(7).

Results. Effect of x-rays on the G-I tract. Following whole body irradiation in the mid-lethal range, rats reduce their food intake by 75% during the first 24 hours. In this same period the stomach becomes greatly distended, and the small bowel loses tone and fills with fluid. During the second 24 hours, anorexia becomes more marked and food intake is reduced by 80-100% of pre-radiation. The obstruction at the pylorus disappears. The food passes into the small intestine, which now becomes distended with fluid and gas. Watery diarrhea usually appears at this time. By the fifth day, the diarrhea subsides, and by the 8th to 10th day the bowel appears grossly normal in surviving animals.

Effect of x-ray on intestinal absorption. Studies were made using groups of 4-6 animals. Control and irradiated groups were handled in an identical manner except for the radiation, and were studied simultaneously in each experiment. Since the absolute values of the dose administered in terms of International Units varied from 20,000-25,000 I.U. in different experiments, the data have been tabulated as percentage of dose. Absorption has been measured in two ways. First, by determining the amount of vitamin A remaining in the gastro-intestinal tract; and secondly, by measuring the amount present in the remainder of the animal. Experiments were made on the 1st, 2nd, 3rd, 4th, 6th and 7th days post-irradiation. After the 6 hour test period, there was less recovered in the intestine of the radiated animals than in the controls, except for one experiment which began 1 hour after irradiation. Vitamin A recovered in the remainder of the animal, except for 2 experiments, also showed greater absorption in the radiated animals. The results of experiments performed on the 3rd day post-irradiation are given in Table I. In the 10 hour test period experiments (Table II) the intestinal recovery values show little difference between the control and irradiated animals. In these 10 hour test period experiments, it has been necessary to base absorption entirely on the amount retained in the intestine since an analysis of the carcass and liver values indicated a mean recovery at 10 hours which was considerably below the carcass-liver recovery at 6 hours in the radiated animals. Differences between the absorption at 6 and 10 hours can probably

7. McCoord, A. B., personal communication.

TABLE II.
Percentage Dose of Vitamin A Recovered 10 Hours After Administration.

Time post-radiation		Intestine	Liver	Carcass	Total recovery
3 days	R*	22.0	6.5		
	C	38.0	17.0		
3 "	R	46.7		11.2	70.6
	C	50.7		5.8	66.4
3 "	R	77.3	8.5	3.2	
	C	59.2	16.2	3.0	
4 "	R	49.7	9.8	4.0	64.2
	C	58.6	13.7	5.0	67.3

* R = Radiated. C = Controls.

TABLE III.
Recovery of Vitamin A in Segments of the Gastrointestinal Tract.

		6 hr vitamin A experiments			10 hr vitamin A experiments		
Days post-radiation		Stomach	Intestine	Total GI tract	Stomach	Intestine	Total GI tract
3 days	R*	52.1	26.2	78.3	28.6	18.1	46.7
	C	56.7	23.5	80.2	30.7	20.0	50.7
3 "	R	37.6	13.8	51.4	47.1	30.2	77.3
	C	44.2	15.5	60.7	46.2	13.0	59.2
3 "	R	29.5	2.5	32.0			
	C	55.8	9.2	65.0			
3 "	R	15.7	13.3	29.0			
	C	40.5	17.5	58.0			
3 "	R	21.5	2.0	23.5			
	C	59.8	2.7	62.5			
4 "	R				38.0	11.7	49.7
	C				41.3	17.3	58.6

* R = Radiated. C = Controls.

be best explained by differences in motility of the gastro-intestinal tract, the controls reaching the same degree of total absorption but at a slower rate. This is shown in Table III where the stomach and intestinal values are separated for the 3 day experiments. In 6 hour experiments, controls showed slower gastric emptying and less absorption. At 10 hours the control stomach values were slightly lower than the radiated.

Effect of diarrhea on absorption. It would seem reasonable to expect reduced absorption during the period of diarrhea. However, both the intestinal recovery values and the carcass values showed an increased absorption in the 3rd and 4th days' experiments when diarrhea was most severe, and the small bowel was flaccid and distended with gas.

Effect of x-ray on the distribution of alcohol vitamin A. The vitamin A content of the liver was lower in the radiated than in the control groups. This was observed even when the recovery from the intestinal tract and liver-carcass showed increased absorption in the radiated animals. In column II of Tables I and II are given the liver values. At the same time the carcass values are markedly increased after the 6 hour test (column III, Table I). The site of deposit of vitamin A in the radiated rats has not been determined. A limited number of studies in which individual organs were studied showed no clear cut shifts. The redistribution, therefore, seems to be widespread and probably indicates a storage of absorbed vitamin A in the peripheral fat deposits. The peripheral vitamin A appears to be rapidly metabolized since the large amounts present at 6 hours in the carcass of the irradiated animals has largely disappeared by 10 hours. Recoveries in the carcasses of both irradiated and control rats are essentially the same at 10 hours.

Effect of x-ray on adrenal vitamin A. A redistribution of vitamin A also occurs in the

TABLE IV.
Percentage Dose Recovered in the Adrenals.

Time post-radiation		6 hr	10 hr
3 days	Radiated	.018	.045
	Control	.023	.013

case of the adrenal gland. At 6 hours the radiated adrenals contain less vitamin A than the controls in the 3 days post-radiation experiments. While at 10 hours the irradiated adrenals contain more vitamin A than the controls (Table IV).

Effect of vitamin A ester. The alcohol form of vitamin A was used in all but one experiment. By using the alcohol the step requiring the hydrolysis of the ester by pancreatic enzymes was eliminated. This simplified the interpretation of the data on absorption. In the one 6 hour experiment where the ester was given to 3 days post-radiation rats, absorption by the radiated animals was slightly lower than that of the controls. The distribution was essentially the same as with the alcohol.

Discussion. Intestinal absorption like many other physiological processes responds to irradiation in a complex manner. From our own studies, and those in the literature, it becomes obvious that a number of variables, such as dosage time and species must be considered in making any statement about the effect of radiation on intestinal absorption. Since the results obtained in this experiment are quite different from those which might be predicted from earlier work, certain fundamental differences between the studies should be pointed out. Most of the earlier work was done with large doses of irradiation limited to the abdomen or a single loop of the bowel. The exact dosages used were not known, although they were in the range which causes an erythema. This resulted in direct irradiation to the intestine with larger doses than we obtained with the whole body radiation doses we have used. The experiments of Buchwald(2), Martin and Rogers (5), and Mottram, Cramer and Drew(9) come in this group. That absorption may be impaired with such large doses seems quite reasonable since very large doses of x-ray will cause irreversible damage to the intestine.

The distribution of vitamin A in tissues indicates that certain major changes in vitamin A metabolism may occur as a result of irradiation, but this is probably not specific since McCoord(8) has described a similar change in rats infected with mouse paratyphoid bacilli, and in rats severely depleted of vitamin A, or subjected to hyperthermia. Liver content is consistently low in the radiated animals and does not support the claim that liver content is an accurate measure of absorption(12). Our studies indicate a rapid destruction of absorbed vitamin A. The radiated carcass values are high at the 6 hour period, and low at 10 hours, and since there are no other sites for storage, the vitamin A had to be either destroyed or transferred back to the intestine. The latter does not seem likely in view of present-day concepts of intestinal function.

The adrenals are known to contain relatively large amounts of vitamin A which presumably participates in the formation of adrenal steroids. In these studies we found evidence of definite disturbances in adrenal vitamin A metabolism. Such findings are not surprising in view of the marked changes in the adrenal reported by Patt(10) after whole body irradiation of rats. However, these changes probably are not specifically related to radiation, since similar changes in vitamin A content have been observed following experimental infection, hyperthermia, chloroform poisoning(8) and after cold stress(11).

Summary. The absorption of vitamin A alcohol by young female rats at varying intervals during the first week following 625 r, whole body irradiation has been studied 6 and 10 hours after a test dose of vitamin A.

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Absorption after a 6 hour test is increased from the 2nd to 6th days in irradiated animals. After a 10 hour test there was little difference between irradiated and control animals. Liver content of vitamin A is decreased in irradiated animals, and there is an increase in carcass vitamin A. Adrenal vitamin A is less in irradiated than control rats at 6 hours and is increased above control rats at 10 hours.

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Presence of Desoxyribonuclease Activity in Human Serum.* (17933)

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Greenstein and Jenrette(1) studied the depolymerization of thymonucleic acid by extracts from various tissues and body fluids. The enzyme responsible for this depolymerization was found to be present in the serum of the guinea pig, mouse, rabbit and dog, but not in that of man. McCarty(2) isolated the enzyme from beef pancreas, termed it "desoxyribonuclease", showed that it was activated by magnesium, and found that the optimal concentration for such activation was 0.003 M. In view of McCarty's findings, it was of interest to determine whether the addition of magnesium ion to human serum would reveal any desoxyribonuclease activity and, if so, to study this activity in normal individuals and in patients with cancer and noncancerous disease.

Desoxyribonucleic acid was prepared from calf thymus, essentially in accordance with Hammarsten's method(3), and dried in vacuum to constant weight. Analyses gave a value of 8.11% for the phosphorus content. The desoxyribonucleic acid was dissolved in 0.028 M veronal buffer of pH 7.5 so as to yield a concentration of 0.8 mg desoxyribonucleic acid per cc. Batches of this solution were filtered just before each series of determinations. McCarty's method for determination of the desoxyribonuclease activity was employed. A volume of 4.0 cc of the 0.08% solution of desoxyribonucleic acid was placed in an Ostwald viscosimeter immersed in a water bath at $37.0 \pm 0.1°$. The relative viscosity, η, of this solution was usually between 2.80 and 3.00. When this solution had attained the temperature of the bath, any substance such as Mg^{++} ion, the effect of which was being investigated, was added. The contents were mixed thoroughly by raising and lowering the solution several times, and several viscosity determinations were made in order to obtain an initial value. At a stated time, 1 cc of serum, brought to 37.0°, was added; the contents were mixed again and the change of viscosity with time was determined. In accordance with McCarty's procedure, the slope of the straight line portion of this curve was taken as the reaction velocity. In this paper, the velocity is expressed as the change in the relative viscosity, η, per 10^3 seconds.

Effect of Mg^{++} concentration. One-tenth cc of distilled water, or of various concentrations of Mg^{++}, was added to 4.0 cc of the desoxyribonucleic acid solution, and 1.0 cc of a pooled specimen of serum was then added. Table I shows that in agreement with the observations of Greenstein and Jenrette, only very slight activity was demonstrable in human serum in the absence of added magnesium over a period of several hours. If the reaction was allowed to proceed overnight (20 hours) measurable activity was manifest. The addi-

* This investigation was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

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TABLE I.
Effect of Mg^{++} Ion on Desoxyribonuclease Activity of Human and Animal Sera.

Final concentration of added Mg, M	Velocity			
	Human	Guinea pig*	Rabbit	Dog
.000	.023	.033	.305	.210
.0002	.059	.193	.370	.255
.003	.150	.345	.800	.301
.010	.098	.315	.790	.238
.020	.066	.280	.520	.190

* Undiluted guinea pig serum, even without the addition of Mg^{++}, gave such high activity that it could not be precisely measured. Consequently, 1 cc of a 1:5 dilution was used in these experiments.

TABLE II.
Thermolability of Human Serum Desoxyribonuclease Activity.
Serum heated for 20 min. at 55° to 60°. Final concentration of Mg^{++} was 0.003 M. Other conditions as described in text.

	Activity of unheated enzyme	Activity of heated enzyme	Decrease in activity, %
Serum 1	.180	.006	96
" 2	.150	.015	90

tion of magnesium chloride caused an activation of the desoxyribonuclease, sufficiently marked so that considerable changes in viscosity were obtained within one to 2 hours. The magnesium ion concentration necessary for optimal activation of human serum was found to be 0.003 M. Depolymerization of thymonucleic acid by the enzymic activity of the serum of the guinea pig, the rabbit, and the dog has been previously demonstrated (1). However, as shown in Table I, the addition of magnesium ion caused an increase in the desoxyribonuclease activity of all three sera. The concentration of magnesium ion necessary for optimal activity of guinea pig, rabbit, and dog sera was similarly found to be 0.003 M.

Thermolability of human serum desoxyribonuclease. In order to confirm the enzymic nature of the action, human serum was divided into 2 portions, one of which was kept at room temperature while the other was heated in a water bath at 55° to 60° for 20 minutes prior to determination of its activity. Table II shows that 90 to 95% of the enzymic activity was lost as a result of heating the human serum under the conditions described. This finding is in essential agreement with that of McCarty's observations on beef pancreas desoxyribonuclease.

Effect of pH. McCarty observed that the optimum pH for the action of beef pancreas desoxyribonuclease covered a broad range from 6.8 to 8.2. By using, as McCarty had done, a solution of desoxyribonucleic acid in veronal buffer of pH 7.5, we obtained, after the addition of human serum and at the end of the determination, pH values which, in practically all instances, lay between 7.50 and 7.80. Several series of determinations in quintuplicate showed that, within experimental error, there was no effect of pH upon activity within this pH range.

Desoxyribonuclease activity of serum in normal individuals, in patients with noncancerous disease, and in patients with cancer. In order to facilitate comparison, the desoxyribonuclease activity of the serum may be expressed in terms of units, where 1 unit is defined as a change of 1.0 in the relative viscosity produced in 10^3 seconds at 37° by adding 1 cc of serum to 4 cc of a 0.08% solution desoxyribonucleic acid in 0.028 M veronal buffer of pH 7.5. The control group consisted of sixteen apparently healthy individuals who ranged in age from about 21 to 65 years, and averaged 30 years. They showed serum desoxyribonuclease activities ranging from 0.14 to 0.54 unit and averaging 0.30 unit (Table III). The activities were not related to sex. The sera of 34 patients not having cancerous disease were tested for desoxyribonuclease activity.† This group ranged in age from 14 to 86 years and

† We are indebted to Dr. Benjamin Shafiroff of the New York University Surgical Division, Goldwater Memorial Hospital, for his cooperation.

TABLE III.
Mean Value and Distribution of Serum Desoxyribonuclease Activity in Normal Individuals, Patients with Noncancerous Disease, and Patients with Cancer.

Group	No. of cases	Avg value in units	% of cases having activities, in units, of			
			.00-.100	.101-.200	.201-.300	>.300
Normal individuals	16	.30	0	25	31	46
Noncancerous patients	34	.21	6	35	50	9
Patients with cancer	50	.15	31	52	10	6

The t value for the difference between the normal individuals and noncancerous patients was 3.2; for that between the normal individuals and cancerous patients, 5.7; and for that between the noncancerous and cancerous, 3.3.

averaged 50 years, and included a wide variety of noncancerous disease such as rheumatic fever, arthritis, cirrhosis, heart disease, benign tumors, diabetes, tuberculosis and diseases of the urogenital tract. The desoxyribonuclease activities ranged from 0.03 to 0.49 unit and averaged 0.21 unit. This average value was significantly lower than that for the group of normal individuals ($t = 3.2$; $P = <0.01$).

The group of 50 cancerous patients included cancers of the gastrointestinal tract, breast, genitourinary tract, Hodgkin's disease, melanoma, and liposarcoma. The ages in this group ranged from 24 to 75 years and averaged 52 years. The serum desoxyribonuclease activities ranged from 0.02 to 0.38 unit and averaged 0.15 unit. This average value was significantly lower than that for the group of normal individuals ($t = 5.7$; $P = <0.01$), and also than that for the group of patients with noncancerous disease. ($t = 3.3$; $P = <0.01$).

The distribution of the desoxyribonuclease activities in the three groups is of interest (Table III). Thus, the incidence of activities less than 0.10 unit in the normal, noncancerous, and cancerous groups were 0, 6, and 31%, respectively, of the individuals in each group. 83% of the patients with cancer had activities less than 0.20 unit whereas only 41% of the patients with noncancerous disease and 25% of the normal individuals had activities below this level.

Discussion. The desoxyribonuclease activity of the serum has hitherto received scant attention. Because the activating effect of magnesium ion on this serum enzyme has not been recognized, it has been held that this enzyme is not present in human serum. The present work shows that the average serum desoxyribonuclease activity in the group of patients with cancer was significantly lower than the average in the normal group or in the group with noncancerous disease. The incidence of decreased serum desoxyribonuclease activities in the group of cancer patients does not appear to be sufficiently high to be of diagnostic value. No explanation for the occurrence of decreased serum desoxyribonuclease activity in disease can be offered at present. In general, such a decreased activity may reflect diminished output of the enzyme from one or more tissues of the body or the increased production of an inhibitor. Further study of the factors controlling the desoxyribonuclease activity of human serum is necessary before these possibilities can be evaluated.

Summary. The addition of Mg^{++} in a final, optimal, concentration of 0.003 M to human serum leads to distinct and readily measurable desoxyribonuclease activity. The average value of this activity in a group of fifty patients with cancer was significantly lower than the average value in a group of 34 patients, of comparable age, with noncancerous disease. Both average values were less than that of a normal group.

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A Colorimetric Procedure for the Determination of Aspartic Acid.* (17934)

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Although there are many procedures in the literature for the determination of aspartic acid it now seems certain that classical isolation methods have yielded low results(1). Microbiological assays are specific for the L isomer only(1). Braunstein's(2) modification of Dakin's(3) method which is specific, accurate, and non-selective with respect to the D and L forms, is based on the stoichiometric conversion of aspartate to fumarate (2,3). Fumaric acid is then reduced to succinate and the latter is measured with succinic dehydrogenase. The present report describes a procedure which eliminates this reduction and enzymatic assay and analyzes colorimetrically the fumaric acid produced from aspartic acid with dimethyl sulfate. The determination is applicable to amino acid mixtures and hydrolysates when the amount of aspartic acid measured exceeds 0.5 mg. Asparagine interferes with this analysis as with the enzymatic method.

Procedure. One ml of aqueous solution containing from 0.6 to 1.4 mg of aspartic acid was pipetted into a 20 ml beaker and made alkaline with sodium hydroxide. Alternately methyl sulfate (Daigger and Company, Chicago, Ill., Highest Purity) and 33% sodium hydroxide were added dropwise to the mixture over a one hour period. The final volume of methyl sulfate and sodium hydroxide added was 0.25 ml each. The solution at no time became acid. Between the additions the beaker was agitated on the Dubnoff metabolic shaker. It was placed in a refrigerator overnight and on removal the solution was adjusted with 10 N sulfuric acid to approximately pH 2. The acid contents were mixed with 1 g of dry silica, prepared as described by Marshall, Orten and

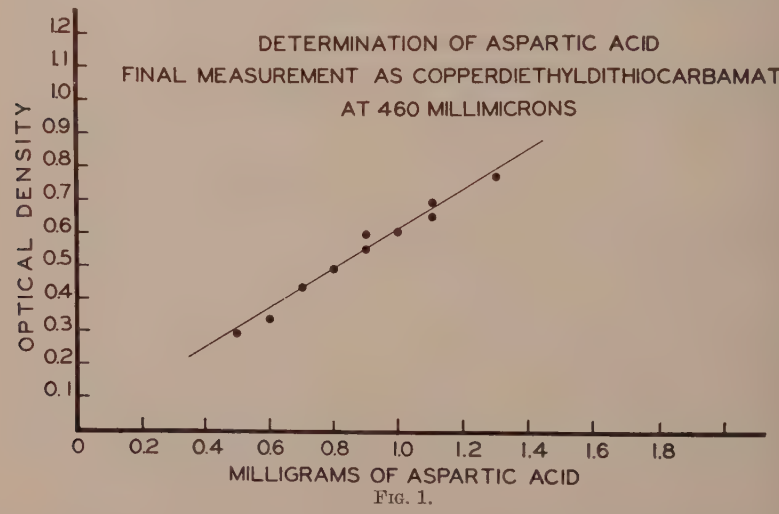

FIG. 1.

* Supported by a grant from the Damon Runyon Memorial Fund.

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TABLE I.
Analysis of Aspartic Acid in Protein Hydrolysates.

Protein	Nitrogen, %	Aspartic acid, % Observed	Aspartic acid, % Literature
Crystalline lysozyme	16.90‖	10.60†	10.2 (6)
Crystalline bovine serum albumin*	15.75§	9.92‡	10.25 (7, 8) 10.9 (9) 11.1 (10)

* Kindly supplied by Dr. Max S. Dunn.
† Average of 3 determinations.
‡ " " 2 "
§ Corrected for moisture and ash (moisture, 1.84%; ash, 0.55%).
‖ Moisture and ash not determined.

TABLE II.
Recovery of Aspartic Acid from Amino Acid Mixtures.

Determination No.	Synthetic mixture* Added, mg	Found, mg	Recovery, %	Casein hydrolysate† Added, mg	Found, mg	Recovery, %
1	.99	.89	90	0	0.67	
2	.99	.96	97	.93	1.59	99
3	.99	.94	95	.93	1.66	107
4				.47	1.16	104
Avg			94			103

* Per 100 ml of solution the mixture was composed of DL-tyrosine 10.3, tryptophane 2.2, cystine 1.4, lysine 2.1, arginine 2.7, glutamic acid 18.6 mg.
† The casein contained 13.4% N and 6.75% of aspartic acid.

Smith(4), suspended in 15 ml of 25% (volume/volume) butanol in chloroform and filtered through paper. After the residues were washed with 20 ml of the butanol-chloroform mixture the filtrate and combined washings were dried in a current of air at room temperature. The dry residue of fumaric acid was dissolved in 1 ml of warm water (70°) and transferred to a centrifuge tube with two 0.25 ml washings. The fumaric acid was precipitated as the copper-pyridyl complex(5) and the copper thus combined was determined colorimetrically(5). The calibration curve is shown in Fig. 1. The Beckman, Model DU, was employed for the photometric measurements.

Hydrolysates. Crystalline bovine serum albumin and crystalline lysozyme were refluxed for 20 hours with 10 times their weight of 6 N hydrochloric acid. When less than 0.8 mg of aspartic acid was assayed, the formation of the copper-pyridyl fumarate, in the final measurement, was favored by scratching the inner surface of the centrifuge tube with a small glass rod. Application of the procedure to hydrolysates provided the data in Table I.

Recovery studies. The analysis of the amino acid in mixtures is shown in Table II. Two amino acid preparations were used. One was synthetic; the other was a hydrolysate of casein. In 8 other samples, each containing 0.97 mg of aspartic acid by weight, the average amount found was 0.93 mg, the standard deviation ±0.06 mg.

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Discussion. The foregoing procedure is recommended for the determination of aspartic acid in protein hydrolysates. Biological fluids in general cannot be measured because of possible interference by fumaric acid. Although the use of the chromatographic determination for fumaric acid(4) would improve the sensitivity, a prior analysis of fumaric acid in the sample would decrease the convenience of the method. Obviously, the precision, specificity and sensitivity of the final measurement can be no greater than that reported for the conversion(3) and the colorimetric procedure for fumarate(5).

Summary. A procedure is described for the determination of aspartic acid in quantities of 0.5 mg or above in the sample. The amino acid is converted to fumaric acid and this is measured colorimetrically.

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Methylene Blue, 2,4-Dinitrophenol, and Oxygen Uptake of Intact and Homogenized Embryos.* (17935)

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That M.B. and 2,4-D.N.P. in suitable concentrations stimulate or augment the oxygen consumption of intact cells and tissues of a variety of biological forms seems well established(1,2,3). The exact mechanisms involved in such reactions, however, do not seem to be so well understood and many ideas have been advanced in attempting explanations of such phenomena. More recent investigators of the action of these chemicals upon the oxygen uptake of intact and homogenized cells emphasize the importance of factors associated with cell structure in conditioning the results(1,4). Marked effects upon intact cells have been pointed out while quite opposite and at times contrary results are described for homogenates and intracellular constituents of the same materials(1). Additions of various substrates to homogenates in certain instances seem to condition or at least influence the nature of the response(1,4).

Since much of the available data seems to have been obtained for mammalian tissues requiring the addition of substrates for the reaction it was thought desirable to extend these observations to the embryonic cells of invertebrates which normally do not require the addition of substrates for such studies. The intact embryo of the grasshopper, *Melanoplus differentialis,* which has proven of value for various cellular problems, has been used in the present investigation(2,3). Homogenates as well as intracellular constituents (nuclei, mitochondria, microsomes) prepared by fractional centrifugation techniques have also been employed. The suspension medium used throughout all experiments was .9% NaCl, buffered to pH 6.8 by .006M phosphates. No additional substrates were added so that oxygen uptake, as measured by standard Warburg manometers, represents that necessary for endogenous respiration. Respiration flasks, 5 ml capacity, were used and all experiments were conducted at 25°C. One hundred embryos per cc of suspension medium or their equivalent were used throughout. Homogenates were prepared by means of an all glass Potter-Elvehjem homogenizer (5). Nuclei, mitochondria and microsomes

* Aided by grant from National Institutes of Health. Acknowledgement is gratefully made to Etta Andrews for technical assistance in carrying out this investigation.

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were separated by means of fractional centrifugation in plastic tubes at speeds of 600 to 1800 times gravity. All other details of technic were as previously indicated(3).

Results. Methylene blue. M.B. has previously been shown to markedly augment the oxygen uptake of actively developing intact grasshopper embryos(2) and as graphically shown in Fig. 1, such results have been confirmed in the present experiments. Maximum stimulation of oxygen consumption for the intact embryo occurs at concentrations of approximately 25 x 10^{-5} M. Homogenates, made from similar embroys, are also stimulated in their O_2 uptake to approximately the same degree by this reagent (Fig. 1). Intact nuclei, separated by fractional centrifugation and thoroughly washed, show a similar augmentation of O_2 uptake when treated with M.B. (Fig. 1). However, when granules (mitochondria and microsomes) are similarly treated, no reaction to M.B. can be detected (Fig. 1). It thus seems that intact embryos and nuclei, as well as the homogenate, respond to M.B. by an augmentation of oxygen consumption while cytoplasm constituents (mitochondria, microsomes) are not so affected.

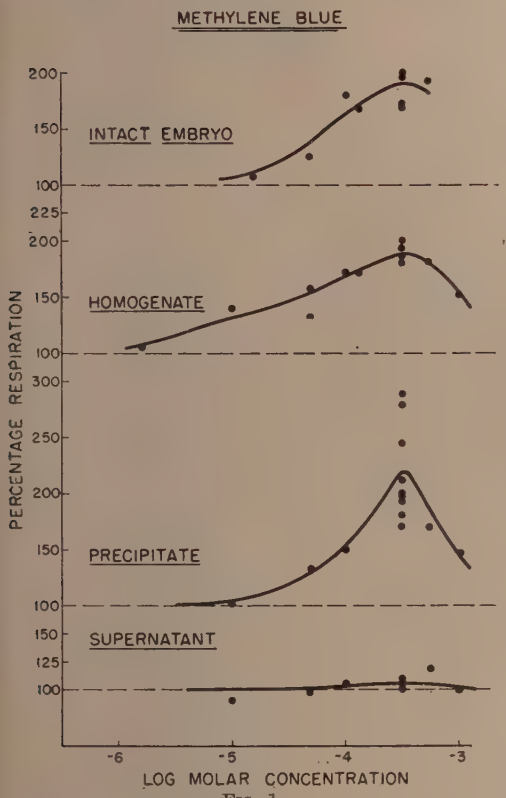

FIG. 1.

Shows effect of M.B. on the oxygen uptake of intact embryos, homogenate, precipitate (nuclei) and supernatant (mitochondria and microsomes). Data expressed as % of normal in .9% NaCl suspension medium taken as 100%. 100 intact embryos/cc of suspension medium: 1 cc of homogenate equal to 100 embryos. Homogenization carried out at room temperature (22-24°C). Equilibration 15 minutes. Readings every 10 min. Values given are averages for 70 min. exposure to reagent and represent readings from a minimum of 12 manometers for each concentration of reagent. Note—All concentrations of M.B. employed give only stimulation. For precipitate (intact nuclei), at conc. of 25 × 10^{-5} M. Note range of results for large series of experiments. Centrifugation carried out at 600 times gravity for nuclei: 1200 times gravity for mitochondria: 1800 times gravity for microsomes. Supernatant used as substrate for mitochondria and microsomes. Washed intact nuclei contain inherent enzyme-substrate system.

2,4-dinitrophenol. As previously noted and confirmed by the present results, 2,4-D.N.P. stimulates oxygen uptake of intact embryos (3) (Fig. 2). Similar concentrations of this reagent when added to homogenates or intracellular constituents (nuclei, mitochondria, microsomes) produce no appreciable or significant effects on their oxygen consumption (Fig. 2). The intact cell or embryo thus seems necessary for the stimulating effects of 2,4-D.N.P. and this is in striking contrast to the action of M.B. However, in the case of M.B. the nuclei, as used, are intact and as such perhaps contribute to the response to the reagent. Since no satisfactory method for breaking the nuclei, without injury to enzyme-substrate system, is at hand this point must await for further elucidation.

Discussion. Present data for the augmentation of the oxygen uptake of the intact grasshopper embryo by M.B. and 2,4-D.N.P. confirm those for this and other biological forms. The marked differences in response of the homogenate and intracellular constituents to M.B. and 2,4-D.N.P. are of some interest in view of the results noted for similar experiments with rat cerebral cortex. Peiss

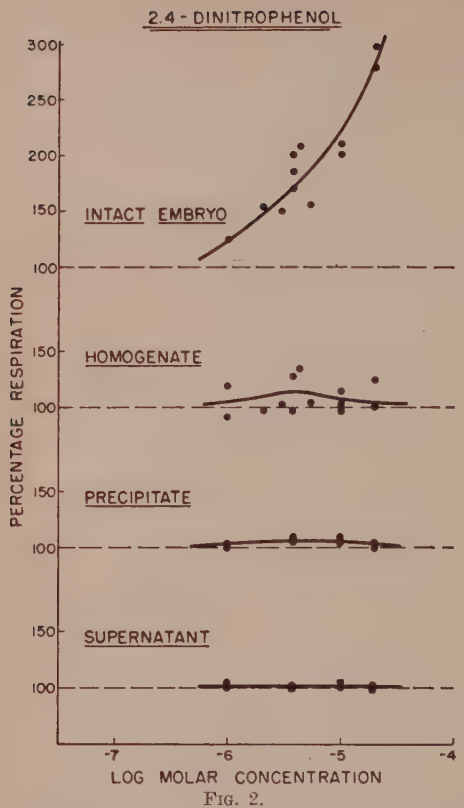

FIG. 2.

Similar to Fig. 1 except for 2,4-D.N.P. Note lack of stimulation of oxygen uptake in all except intact embryos.

and Field(1) have shown that brain slices respond to 2,4-D.N.P. by a marked augmentation of the oxygen uptake while homogenates made from similar tissues show no such reaction. As a matter of fact a rather marked inhibition of oxygen uptake of the homogenate is produced by doses of 2,4-D.N.P. which normally stimulate the intact brain slices. Tyler(4) more recently has pointed out that uninjured, intact nuclei of a rat brain homogenate are necessary for an augmenting effect of oxygen uptake by 2,4-D.N.P. For injured nuclei in the homogenate an inhibitory action is noted when similar concentrations of the reagent are employed. For the homogenate, intact nuclei and intracellular constituents of the grasshopper embryo, no augmenting action of 2,4-D.N.P. has been noted even though cytological examinations have revealed no apparent changes in the nuclei of the homogenate. From such observations one might well infer that for the grasshopper embryo the cell must be intact in order to produce significant augmenting effects of oxygen uptake by 2,4-D.N.P.

Although both M.B. and 2,4-D.N.P. may produce striking augmentation of oxygen uptake by the intact embryo they differ markedly in their action upon homogenates. The augmentating action of M.B. seems to be confined exclusively to the intact nuclei and does not affect the mitochondria or cytoplasmic constituents.

Summary. 1. M.B. and 2,4-D.N.P. produce augmentation of oxygen uptake of intact grasshopper embryos. 2. M.B. produces augmentation of oxygen uptake of homogenates made from embryos. 2,4-D.N.P. does not produce significant augmentation of oxygen uptake of homogenates. 3. M.B. produces augmentation of oxygen uptake of intact nuclei and not of mitochondria or cytoplasmic constituents. 4. 2,4-D.N.P. does not affect oxygen uptake of isolated nuclei or mitochondria or other cytoplasmic constituents.

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Metabolism of C^{13}-Carboxyl-labeled Malonate by the Intact Mouse* (17936)

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There are a number of papers in the literature which deal with metabolic relationships of malonate other than the inhibition of succinic dehydrogenase. Thus, additional enzymatic inhibitory effects have been suggested(1-3) for this compound. A stimulating effect on the oxygen uptake and carbon dioxide output of sea urchin eggs has been described(4). Malonate has been reported isolated, presumably as a metabolic product, from mold and bacterial cultures(5-8), as well as utilized by such micro-organisms(7,9). According to Vennesland and Evans(10), malonic acid is a product of the metabolism of oxalacetate by a mammalian (pig) heart preparation. In the present investigation the conversion of the carboxyl carbon of malonate to carbon dioxide has been studied in the intact mouse and in dog plasma. The results are interpreted to indicate the probable occurrence in mammalian cells of enzymatic mechanisms for conversion of malonate to carbon dioxide.

Methods. C^{13}-carboxyl-labeled malonic acid containing 7.54 atom % excess C^{13} was synthesized according to the method of Calvin, *et al.*(11). The melting point and acid value of the synthetic material were the same as those of recrystallized Eastman malonic acid. For administration the acid was neutralized with sodium hydroxide and made just colorless to phenolphthalein with 0.1 N HCl.

Experiments on mice. Unfasted mice of the A strain were employed. Malonate (about 1 mM per 100 g of body weight) was injected in a total volume of 1 ml into 3 to 4 subcutaneous sites on the back of the animal. The mouse was placed immediately in a system for collecting the respiratory carbon dioxide in sodium hydroxide. The total carbon dioxide in the alkali was measured by means of the Van Slyke manometric apparatus. C^{13} concentration was determined mass spectrographically. The dose of malonate employed is of the order of ½ to ¼ of the fatal dose for the mice in question. The only effect on the behavior of the animals noted was the production of a degree of lethargy in certain of the mice.

Experiments on dog plasma. Into each of 2 Warburg type flasks of about 170 ml capacity were placed 25 ml of dog plasma plus .25 mM of C^{13}-malonate, and the vessels were shaken at 38°C for 5 hours. At the end of this period, the flask contents were acidified with strong sulfuric acid and the carbon dioxide absorbed into sodium hydroxide for subsequent isotope analysis. The gas phase of one flask was air; of the other, 5% carbon dioxide in oxygen.

Results and discussion. Experiments on mice. In Table I it will be noted that 20-32% of the administered C^{13} was recovered in the respiratory carbon dioxide within 6 hours after injection. Approximately 10% of the administered dose was accounted for in the first hour. The corresponding values decrease with time until, for 2 out of 3 animals, the carbon dioxide in the 6th hour contained

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10. Vennesland, B., and Evans, E. A., Jr., *J. Biol. Chem.*, 1944, v156, 783.
11. Calvin, M., and others, Isotopic Carbon, 1949, p. 192, John Wiley and Sons, Inc.

TABLE I.

Recovery of C^{13} in Respiratory CO_2 After the Subcutaneous Injection of Approximately* .25 mM of Carboxyl-labeled Malonate Containing 7.54 Atom % Excess C^{13} in the Whole Molecule.

Mouse Sex, wt in g	1 ♀, 26	2 ♀, 25			3 ♀, 25			4 ♂, 25		
		C^{13}			C^{13}			C^{13}		
CO_2 collection in hr	Total CO_2 mM	Total CO_2 mM	Atom % excess	% of admin. dose	Total CO_2 mM	Atom % excess	% of admin. dose	Total CO_2† mM	Atom % excess	% of admin. dose
1st	3.67	2.60	.20	9.9	3.93	.15	10.9	3.40	.15	10.
2nd and 3rd	5.41	5.38	.14	14.4	6.53	.06	7.2	5.19	.08	8.2
4th and 5th	5.22	4.09	.08	6.2	5.40	.02	2.	4.81	.02	1.9
6th	2.15	1.70	.06	1.9	2.16	.01	.0	2.12	.01	.0
25th	—	—	—	—	3.28	.00	.0	—	—	—
Total				32.4			20.1			20.1

* Mice 2, 3, and 4 received .23, .24, and .22 mM of malonate, respectively. Mouse 1 received no malonate.

little, if any, labeled carbon. Data for the output of respiratory carbon dioxide of a mouse to which no malonate had been administered are included in Table I to indicate that the carbon dioxide production of the animals receiving malonate was of the same order of magnitude as the normal. It is reasonable to assume that the initial step in malonate metabolism is decarboxylation to acetate. The further reactions of malonate should then be similar to those of acetate, except as the presence of malonate itself has influenced metabolism.†

Experiments on dog plasma. The acid-labile carbon dioxide in each of the 2 flasks contained only .005 atom % excess C^{13}. This value is within the error of the analytical method and indicates little or no decarboxylation of malonate by dog plasma. Given normal plasma bicarbonate levels, decarboxylation of 1% of the added malonate would have been detectable. Hence it appears likely that in the mouse the major site of the conversion of malonate to carbon dioxide is intracellular and that the process is at least in part enzymatic. It is recognized that this inference involves the assumption that the results on dog plasma would hold for not only the extracellular fluids of the mouse, but

† Gould *et al.*‡ have reported that within 4 hours after the intraperitoneal injection of $CH_3C^{14}OOH$ into fasted rats, 87% of the radioactivity was recovered in the respiratory carbon dioxide. In experiments performed after submittal of the present manuscript, recoveries of labeled carbon of the same high order of magnitude were observed in the case of the mice when $CH_3C^{13}OOH$ was administered together with normal malonate. Similar high recoveries might be predicted for any acetate derived metabolically from labeled malonate, if it is assumed, among other things, that the site of malonate decarboxylation is the same as the site of oxidation of exogenous acetate. The finding that only 20-32% of the malonate carboxyl carbon was converted to respiratory carbon dioxide raises the question of the fate of the remaining malonate (urinary excretion, metabolism via pathways not involving acetate, etc.).

‡ Gould, R. G., Sinex, F. M., Rosenberg, I. N., Solomon, A. K., and Hastings, A. B., *J. Biol. Chem.*, 1949, v177, 295.

also the intracellular ones, aside from the enzymatic functions of the latter. Moreover, the degree to which the microorganisms of the gastro-intestinal tract are responsible for the experimental findings has not been evaluated. The parenteral administration of the malonate should reduce the importance of this factor but does not necessarily eliminate it.

Summary. When C^{13} - carboxyl - labeled malonate was injected subcutaneously into mice, 20-32% of the labeled carbon was recovered in the respiratory carbon dioxide in 6 hours.

At body temperature, dog plasma showed little or no ability to decarboxylate malonate.

These results are interpreted to indicate the probable occurrence in mammalian cells of enzymatic mechanisms for conversion of malonate to carbon dioxide.

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Antibiotics in the Treatment of Experimental Acute Hemorrhagic Pancreatitis in Dogs. (17937)

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In a study on the pathogenesis of acute pancreatitis Dragstedt, Haymond, and Ellis (1) reported that devitalized dog pancreas, highly toxic when allowed to autolyze within the peritoneal cavity, was not toxic after being sterilized in the autoclave. Also, sterile fetal pancreas was not toxic when placed within the dogs' peritoneal cavity. Within the uncontaminated pancreas of 13 out of 17 dogs, bacteria common to the intestinal tract were found. *B. welchii* was especially common. No matter what the cause of the necrosis in acute pancreatitis might be, these authors felt that the resulting toxemia was the same and in the major part believed that it was due to the bacterial decomposition products of proteins. These observations suggested to us that antibiotics might be of value in treating experimental pancreatitis in dogs.

Methods. Healthy, adult, dewormed, mongrel dogs were used. Pancreatitis was produced aseptically in the same manner in all the dogs by injecting the animal's own gallbladder bile into its major pancreatic duct and then tying the duct. To produce an hemorrhagic, necrotic pancreatitis with a high fatality rate in non-treated animals it was found necessary to inject a relatively large amount of bile under pressures much higher than have been found normally in the biliary tract of the dog(2). An average of 10.5 cc of bile were injected into the main pancreatic duct extraduodenally through a 20-gauge needle within less than one minute. Pressure readings taken with a mercury manometer attached to a side arm showed pressures of 400 to 500 mm of mercury during the injection. After a satisfactory injection, the pancreatic parenchyma would be stained diffusely; often there would be a mottled brown appearance as described by Mann and Giordano(2).

The animals treated with penicillin were given 300,000 units of penicillin G in 1 cc of oil and wax* intramuscularly, beginning within 4 hours after surgery and continuing once daily for 4 or 5 days. Streptomycin hydrochloride* was given subcutaneously in doses of 0.17 g beginning within 4 hours after the pancreas was injected and continuing every 4 hours, except for the 4:00 A.M. dosage, for

1. Dragstedt, L. R., Haymond, H. E., and Ellis, J. C., *Arch. Surg.*, 1934, v28, 232.

2. Mann, F. C., and Giordano, A. S., *Arch. Surg.*, 1923, v6, 1.

* Provided by E. R. Squibb and Sons, New York City.

TABLE I.
Survival of Dogs with Experimental Pancreatitis.

Type	No. of dogs	Survivors	Deaths
1. Penicillin treatment postoperatively	31*	18	13 (42%)
2. Streptomycin treatment postoperatively	6	3	3
3. Penicillin and streptomycin treatment postoperatively	5	3	2
4. Intestinal "sterilization" preoperatively with streptomycin, sulfasuxidine, or both	10	3	7
5. Pancreatectomy after development of pancreatitis	7	1	6
6. Controls	23†	5	18 (78%)

* 9 had 100,000 units of penicillin intraperitoneally at the time pancreatitis was produced and 6 of these had one injection of penicillin preoperatively. Five others were explored during the first few days postoperatively.

† 2 dogs were explored in the immediate postoperative period.

4 or 5 days. The animals given sulfasuxidine, streptomycin or both preoperatively in order to reduce the intestinal flora received 3 g of sulfasuxidine orally twice a day or 0.5 g of streptomycin orally twice a day for 4 to 5 days before pancreatitis was produced. When a total pancreatectomy was used as a method of treatment it was done aseptically through the old incision under sodium pentobarbital anesthesia within 30 hours following production of the pancreatitis.

Experiments and results. Results in the 6 series of animals used are summarized in Table I. The animals that died of acute pancreatitis succumbed within 4 days postoperatively and usually within the first 48 hours. At autopsy there was a generalized peritonitis with extensive fat necrosis in the upper abdomen and marked swelling of the pancreas with hemorrhagic areas and occasional frank necrosis. When autopsied the survivors usually had a shrunken cord-like pancreas with some nearby adhesions but no other abnormalities.

Those treated with penicillin in Series I had a significantly higher survival rate than the control animals which were treated the same way except for the penicillin. Using a modification of the χ^2 test for 4-fold tables (3) the probability of the differences noted arising through the errors of random sampling is .008. If the animals given penicillin preoperatively and intraperitoneally as well as those explored postoperatively are omitted from consideration, the difference between the fatality rates among those treated with penicillin postoperatively and the controls is still significant with a $P = .029$. The animals treated with streptomycin or a combination of penicillin and streptomycin also seemed to have a higher survival rate than the controls but the series are probably too small to be significant. Attempts to reduce the bacterial flora of the intestinal tract before producing pancreatitis as well as a total pancreatectomy after the development of pancreatitis failed to reduce the fatality rate below that noted in the controls.

Discussion. The effectiveness of parenteral penicillin treatment in reducing the mortality of experimental pancreatitis supports the conclusion of Dragstedt, Haymond, and Ellis(1) that bacteria normally resident in the dog pancreas are important in developing the toxemia of pancreatic necrosis. Similarly the value of penicillin in treating the toxemia following complete hepatic artery ligation(4) or following the intraperitoneal autolysis of detached segments of canine liver(5) has favored the theory that bacteria normally

3. Treloar, Alan E., Elements of Statistical Reasoning, John Wiley and Sons, New York, 1939.

4. Markowitz, J., Rappaport, A., and Scott, A. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 305.

present in the dog liver are important in producing the fatal toxemia of liver autolysis(6).

If it is assumed that the bacteria normally present in the dog pancreas arise from the intestine, reduction of the intestinal bacterial count following oral streptomycin or sulfasuxidine should in time reduce the number of pancreatic bacteria. Effective pancreatic sterilization should then reduce the toxicity of experimental pancreatitis. The failure of these methods to reduce the mortality may be due to an inadequate pancreatic sterilization, for the two animals in the group in which pancreatic cultures were taken before pancreatitis was produced both had gram positive bacteria in the pancreas.

We have no evidence that antibiotics are effective in treating acute hemorrhagic pancreatitis in humans.

Conclusions. 1. Treatment with penicillin G in oil and wax has been effective in lowering the fatality rate of experimental hemorrhagic pancreatitis in dogs. 2. Total pancreatectomy as a treatment or preoperative oral streptomycin or sulfasuxidine as prophylaxis have been ineffective.

5. Lewis, F. J., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 533.

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Pituitary Basophile Hyperplasia and Crooke's Hyaline Changes in Man After ACTH Therapy. (17938)

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(Introduced by P. B. Beeson)

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A significant increase in the basophilic cells associated with Crooke's hyaline cytoplasmic changes was found in the anterior pituitary of two patients following adrenocorticotropic hormone therapy. To our knowledge these findings have not previously been reported in man.

Material. The first patient was a 43-year-old white man with chronic glomerulonephritis who was given a course of ACTH therapy. He died in uremia on the fifth day of treatment having received a total dose of 400 mg intramuscularly. Autopsy confirmed the diagnosis of chronic glomerulonephritis. The combined weight of the adrenal glands was 18.5 g. Histologic examination of the pituitary suggested a distinct increase in basophilic cells, which displayed striking hyaline cytoplasmic changes as described by Crooke in Cushing's syndrome(1). Many of the chromophobe cells showed definite basophilic stippling, suggesting a transition between these and the basophilic cells. Both chromophobes and basophiles were most prominent in the anterior portion of the lobe. No increase in basophiles was noted in the pars intermedia and there was no basophilic infiltration of the posterior lobe.

The second patient was a 19-year-old white nullipara with chronic glomerulonephritis who received 490 mg of ACTH intramuscularly over a period of 8 days, terminating 7 days prior to death in uremia. Autopsy showed chronic glomerulonephritis. The combined weight of the adrenal glands was 18.5 g. Gross examination of the pituitary gland revealed a speckled yellowish gray appearance of the anterior lobe.

Histologic examination suggested a diffuse increase in basophilic cells with a concentration in the anterior and central portions. A minimal basophile infiltration of the posterior lobe was noted. Hyaline cytoplasmic changes of the basophiles were present in only a few scattered cells. Many chromophobes in both

1. Crooke, A. C., *J. Path. and Bact.*, 1935, v41, 339.

TABLE I.
Differential Counts of Pituitaries from 2 Patients Receiving ACTH and from 2 Control Patients.

Patient	Age	Sex	Disease	Total No. cells counted	Acidophile, %	Basophile, %	Chromophobe, %
1 ACTH, 400 mg	43	M	Chron. glom. nephr.	24229	37.9	23.9	38.2
			χ^2		2.75	3582	1484
			p*		>.10	<.01	<.01
Control	46	M	Chron. glom. nephr.	21672	37.8	11.6	50.5
2 ACTH, 490 mg	19	F	Chron. glom. nephr.	21088	44.5	24.5	31.0
			χ^2		.19	9586	2560
			p*		>.50	<.01	<.01
Control	21	F	Acoustic neuroma	21272	44.4	7.2	48.4
Comparison of counts with mean percentages obtained by Rasmussen(2).							
Mean for adult males					37.0	11.0	52.0
Compared with Patient 1				χ^2	61.6	4095	1838
				p*	<.01	<.01	<.01
Mean for nulliparous females					43.0	7.0	50.0
Compared with Patient 2				χ^2	19.6	9895	3042
				p*	<.01	<.01	<.01

* p from the tables of Snedecor, G. S., Statistical Methods, The Iowa State College Press, 1946.

the anterior and intermediate portions revealed basophilic stippling.

Method. Differential counts of the three cell types of the anterior pituitary were performed following the method of Rasmussen (2). The glands were fixed in 10% solution of USP formaldehyde and divided into three blocks by horizontal cuts. In addition to the routine hematoxylin and eosin stain, sections from each block were stained for differential counting by Mallory's 1% acid fuscin, orange G, aniline blue stain(3). A minimum number of 20,000 cells was counted from each gland. Pituitaries from two patients of the same sex, parity and approximate age who had not received ACTH were processed in the same manner and employed as controls. Further details of these cases and the results are shown in Table I.

Results. The basophilic cells in the patients treated with ACTH were increased to 23.9% and 24.5%. This change is statistically significant not only in relation to our controls but also when applied to the large series reported by Rasmussen(2). The increase is associated with a corresponding decrease in the number of chromophobes. These figures, moreover, do not adequately reflect the magnitude of the basophilic change since the many chromophobes with basophilic stippling were not counted as basophiles.

Discussion. These findings cannot be satisfactorily explained at present but a tentative interpretation of their meaning seems warranted. Experimental observations have shown that the administration of exogenous

2. Rasmussen, A. T., *Am. J. Path.*, 1929, v5, 263; *ibid.*, 1933, v9, 459.

3. Mallory, F. B., Pathological Technique, W. B. Saunders Co., Philadelphia, 1938, p. 153.

TABLE II.
Differential Counts of 5 Pituitaries from Hypertensive Patients With or Without Uremia.

Age	Sex	Disease	Total No. cells counted	Acidophile, %	Basophile, %	Chromophobe, %
46	M	Uremia, chronic glomerular nephritis; hypertension	21672	37.8	11.6	50.5
48	F	Essential hypertension, cerebral hemorrhage (nullipara)	23996	45.8	13.5	40.8
84	M	Essential hypertension, nephrosclerosis, bacteremia	20492	46.5	13.0	40.5
58	M	Chronic pyelonephritis, uremia, hypertension	26741	36.4	10.7	53.0
72	M	Essential hypertension, tubular nephritis, uremia	9170	37.6	9.1	53.3
Mean				40.8	11.6	47.6
Stand. dev.				4.9	1.8	6.5

adrenal steroids suppresses the release of ACTH from the pituitary(4). In a patient with unilateral functional carcinoma of the adrenal cortex, atrophy of the other adrenal indicated a similar effect(5). Crooke's changes, which are a constant finding in Cushing's disease, were also present in the pituitary of this patient. The administration of ACTH to our patients may have produced the same effect by increasing the concentration of endogenous adrenocortical hormones. This suggests that basophilism and Crooke's change in our cases may reflect an inhibition of the normal release of ACTH from the pituitary.

It must be considered that the changes which we have described could be related to chronic glomerulonephritis and uremia rather than to the administration of ACTH. Berblinger(6) reports an increase in the basophiles of the anterior pituitary in cases of hypertension, either essential or associated with chronic progressive nephritis and uremia. For this reason we performed differential counts on the pituitaries of five patients who had either longstanding hypertension or chronic progressive nephritis with uremia (Table II). This control series revealed a maximum basophile percentage of 13.5% and a mean of 11.6% which is not significantly different from Rasmussen's mean value of 11%. Rasmussen investigated the pituitaries of 90 patients with hypertension and found no correlation between arterial pressure and the number of pituitary basophiles(7). We wish to reemphasize the need for differential counts in estimating the distribution of cell types in the pituitary since preliminary histologic examination of two cases suggested a definite increase in basophiles which was not confirmed on actual count.

The absence of marked Crooke's changes in the second patient who had not received ACTH during the last 7 days of life, suggests that this change may be readily reversible. In this connection another patient whom we have studied may be of interest. This was

4. Cheng, C.-P., Sayers, M. A., and Sayers, G., *Fed. Proc.*, 1949, v8, 24.

5. Kepler, E. J., Sprague, R. G., Clagett, O. T., Power, M. H., Mason, H. L., and Rogers, H. M., *J. Clin. Endocrinol.*, 1948, v8, 499.

6. Berblinger, W., *Endokr.*, 1935, v16, 19.

7. Rasmussen, A. T., *Endocrinol.*, 1936, v20, 673.

a 19-year-old white nullipara who died of myasthenia gravis. She had received 975 mg and 500 mg of ACTH 9 and 6 months respectively before death. The pituitary showed a focal increase of basophiles, resembling an adenoma. Differential counts done in a similar manner as described revealed 13.9% of basophiles, a few of which showed Crooke's changes. This focal adenomatoid accumulation of basophiles might represent a residual effect of ACTH therapy.

The therapeutic administration of ACTH may stimulate the adrenal cortex which then produces storage of endogenous ACTH in the anterior pituitary. The morphologic expression of this storage appears to be pituitary basophilism and Crooke's changes. Conversely a decrease in basophiles might be expected when the circulating adrenal cortical hormones are reduced and when the pituitary is released from their inhibiting effect. This occurs in Addison's disease where a decrease in the basophiles of the pituitary has been reported(8).

Our findings seem to indicate that morphologic changes are associated with alterations of the functional balance between the anterior pituitary and the adrenal cortex. Such morphologic changes might be useful in the experimental investigation of the relationship between these glands. The advent of ACTH and cortisone makes such an experiment quite feasible.

Summary. The administration of ACTH appears to have produced morphologic changes in the anterior pituitary of two patients. These changes consisted of an increase in the total number of basophiles, Crooke's hyaline cytoplasmic changes in these cells and basophilic stippling of many of the chromophobes. It is possible that these changes reflect the storage of endogenous ACTH following stimulation of the adrenal cortex by the therapeutic administration of this hormone.

8. Crooke, A. C., and Russell, D. S., *J. Path. and Bact.*, 1935, v40, 255.

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Experimental Nephrotoxic Nephritis in the Rat Treated with ACTH or Cortisone. (17939)

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Recent studies have demonstrated the marked effects of ACTH and cortisone on diseases involving hypersensitivity(1,2). The present work was carried out to determine whether experimental nephrotoxic nephritis, which is thought to involve a hypersensitivity reaction, could be prevented by the administration of these two agents.

Methods. Nephrotoxic serum was prepared according to the method used by Smadel (3). Nineteen black and white hooded rats of a specially inbred strain and averaging 250 g in weight, were studied. Weights, eosinophile counts, and white blood cell counts were determined. The urinary sediment was examined and the urinary albumin content was estimated, using both the heat and acetic acid and sulfosalicylic acid methods. Fourteen of these 19 rats received 1 cc of nephrotoxic serum per 100 g of body weight divided into 2 doses intravenously administered on successive days. Six rats

1. Bordley, J. E., Carey, R. A., Harvey, A. M., Howard, J. E., Kattus, A. A., Newman, E. V., and Winkenwerder, W. L., *Bull. Johns Hopkins Hosp.*, 1949, v85, 396.

2. Berthrong, M., Rich, A. R., and Griffith, P. C., *Bull. Johns Hopkins Hosp.*, 1950, v86, 131.

3. Smadel, J. E., *J. Exp. Med.*, 1936, v64, 921.

TABLE I.
Outline of Results.

Material inj.	Rat No.	Alb'uria—days after 1st inj. 0	1	2	3	4	Hist.	Ed. and Asc.
Normal	C-1	0	0	0	0	0	0	0
rabbit	2	0	0	0	0	0	0	0
serum	3	0	0	0	0	0	0	0
NTS	N-1	0	0	2+	2+	4+	2+	0
alone	4	0	3+	3+	4+	4+	2+	0
	5	0	1+	2+	2+	2+	2+	0
	6	0	—	2+	2+	3+	2+	0
	8	0	2+	4+	4+	4+	2+	0
	9	0	2+	4+	4+	4+	2+	0
NTS +	A-1	0	2+	4+	4+	4+	2+	1+
ACTH	2	0	2+	4+	4+	4+	2+	1+
	3	0	2+	4+	4+	4+	2+	1+
	4	0	3+	4+	4+	4+	3+	4+
	5	0	2+	4+	4+	4+	3+	4+
ACTH	AC-1	0	0	0	0	—	0	0
alone	2	0	0	0	0	—	0	0
NTS +	CO-1	0	2+	4+	4+	4+	3+	4+
cortisone	2	0	2+	4+	4+	4+	2+	1+
	3	0	2+	4+	4+	4+	2+	1+

Alb'uria = Albumin in urine, graded from 0 to 4+.
Ed. and Asc. = Degree of edema and ascites found post-mortem, graded from 0 to 4+.
Hist. = Degree of renal damage estimated by histological examination, graded from 0 to 4+.
NTS = Nephrotoxic serum.

(N) received no further treatment. Five rats (A) were given ACTH before the first injection of nephrotoxic serum. Three of these (A-1, A-2 and A-3) received an effective dose of .4 mg of ACTH (Armour, Lot 77-S) per 100 g of body weight every 6 hours starting 9 hours before the first injection of NTS and continuing throughout the experiment. This dose of ACTH did not produce a drop in eosinophile count. Two additional rats (A-4 and A-5) received increasing doses of ACTH (Armour, Lot 44A, 2 & 3) starting at 0.8 mg per 100 g every 6 hours subcutaneously and increasing daily to reach 3 mg per 100 g every 6 hours on the fourth day. Only after this large dose was given did the eosinophiles decrease to 0 and remain there for the duration of the experiment. The first injection of nephrotoxic serum was given to these two rats following the eosinophile decline on the fourth day. The remaining 3 rats (CO) of the 14 that were given nephrotoxic serum, were treated with cortisone acetate (Merck) 5 mg per 100 g twice a day subcutaneously for 24 hours prior to administration of the nephrotoxic serum and for the remainder of the experiment. This dose was sufficient to cause the eosinophile count to drop from the normal range of 2-400 to 0-40 cells per cmm. All of these animals were sacrificed 4 days after the first injection of nephrotoxic serum. Their organs were fixed in formalin and examined by routine histological technics.

An additional control group of 3 rats (C) was given normal rabbit serum in a manner similar to the previous groups. A further control group (AC) was treated with ACTH alone, 0.4 mg per 100 g subcutaneously every 6 hours for 4 days before sacrifice.

Results. The results, summarized in Table I, show that the animals treated with ACTH and cortisone developed the same or an even slightly greater degree of nephritis than those given nephrotoxic serum alone. All of the animals given nephrotoxic serum showed im-

mediate marked albuminuria. There were no erythrocytes, but a few hyaline casts were found occasionally in the urinary sediment. The histological picture of the kidneys was uniformly that of a moderate degree of acute glomerulonephritis, with increased cellularity, avascularity and swelling of the basement membrane of the glomerular tufts. The kidneys of rats A-4 and A-5 showed slightly more severe glomerular involvement, and the tubules in these two rats were moderately dilated. All the rats given ACTH and cortisone showed some edema and ascites, which was very marked in rats A-4, A-5 and CO-1. In these 3 rats the spleen was greatly decreased in size.

Discussion. The present findings are similar to those recently reported by Knowlton, *et al.*(4), who found that cortisone did not prevent the development of cytotoxic serum nephritis in the rat. Our data further indicates that ACTH, in the large dosage necessary to give a continued depression in the eosinophile count, also did not prevent the lesions from developing. ACTH has been shown to affect the experimental cardiovascular lesions in rabbits produced by anaphylactic hypersensitivity to horse serum(2). The failure to prevent the renal lesions produced by the injection of nephrotoxic serum suggests that, in this condition, a different mechanism may be involved.

Conclusion. Both ACTH and cortisone failed to inhibit the development of renal lesions in the rat produced by the injection of rabbit anti-rat kidney serum.

We are indebted to Armour and Company, through the courtesy of Dr. Edwin E. Hays, for generously supplying the ACTH.

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Distribution of Radioiodine in Erythrocytes and Plasma of Man.* (17940)

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Abundant data concerning the distribution of chloride and bromide between the erythrocytes and plasma are available. However, there is a lack of similar data on the distribution of iodide, owing to the difficulties in the chemical determination of iodine. Because of the ease with which I^{131} may be determined we have reinvestigated with the aid of radioiodine, the distribution of iodide in the cells and plasma of man.

Experimental. Venous blood was drawn from normal individuals and immediately heparinized. Various amounts of labeled sodium iodide in a volume of 0.1 to 0.3 cc were added to 25 cc of blood. This volume ratio was chosen so as to disturb the osmotic relationship to a minimal degree. The tubes containing the blood were then rotated for 20 minutes to obtain complete mixing. An aliquot was transferred to a Wintrobe tube for the determination of the corpuscular volume percentage (hematocrit) by means of centrifugation in the cold for 2 hours at 3,000 revolutions per minute. Another aliquot of the blood was taken for analysis of radioactivity by the method previously described (1) and for determination of chloride according to a modification of the method of Keys (2). The remainder of the blood was centrifuged to obtain plasma, in which the I^{131} and chloride concentrations were also determined.

* Abridgment of part of a thesis submitted by Dr. Rall to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medicine.

1. Rall, J. E., Johnson, H. W., Power, M. H., and Albert, A., unpublished data.

2. Keys, A., *J. Biol. Chem.*, 1937, v119, 389.

TABLE I.
Ratio of Iodide and Chloride Content of Erythrocytes to That of Plasma.

Exp.	I^{127}, μg per 100 cc	Chloride		Iodide*	
		Cell: plasma ratio	Cell: plasma ratio on basis of water content	Cell: plasma ratio	Cell: plasma ratio on basis of water content
1	35	.54	.77	.80	1.14
2	3000	.58	.83	.64	.92
3	70	.57	.82	.67	.96
4	20	.62	.90	.66	.94
5	20	.55	.79	.71	1.02
6	35	.57	.82	.65	.92
7	35	.66	.95	.60	.87
8	20	.68	.97	.69	.99
9	60	.53	.76	.65	.92
10	20	.58	.83	.65	.92
Mean		.59	.84	.67	.96
Stand dev.		.050		.054	

* 0.001 microcurie of I^{131} per cc of whole blood.

The iodide and chloride content of the erythrocytes was calculated from the values for whole blood and plasma according to the following formula:

Erythrocyte halide = [whole blood halide—(plasma halide × plasma fraction)]/erythrocyte fraction.

Chloride determinations were done in duplicate, and radioiodine determinations were done in quadruplicate.

Results. The results of 10 determinations of the ratio of the chloride and iodide content of erythrocytes to that of plasma are shown in Table I. Concentrations of sodium iodide ranging from 20 to 3,000 μg per 100 cc were used so as to avoid adsorption errors. It can be seen that the ratio of the iodide content of erythrocytes to that of plasma was somewhat higher than the corresponding ratio for chloride content. When the results were calculated on the basis of cell water content of 65% and plasma water content of 93%, the distribution of iodide between cells and plasma was approximately unity.

Discussion. The results given in Table I imply that the distribution of iodide is different from the distribution of chloride. Weir and Hastings(3) found that the distribution ratios of chloride and bromide were approximately the same, the cell : plasma ratios on the basis of water content being 0.72 and 0.76, respectively. The reason for the difference in behavior of iodide is not apparent. Although anaerobic handling of the blood was not attempted, escape of carbon dioxide with attendant shift of halogen from cells to plasma was minimized as much as possible by filling the tubes completely, using tight stoppers and carrying out the procedure as rapidly as possible. Since each set of analyses for chloride and radioactive iodide was made on aliquots of the same plasma, errors attributable to slight shifts in halogen equilibrium may be assumed, at least provisionally, to have affected the distribution ratios of chloride and iodide equally and in the same direction. In so far as this assumption is justified, the data indicate that a portion of iodide added to human blood is not freely diffusible and may be bound in some manner within the erythrocytes.

Summary. The ratio of chloride content of the erythrocytes to that of the plasma was found to be 0.59, and the corresponding ratio for radioiodide was 0.67.

3. Weir, E. G., and Hastings, A. B., *J. Biol. Chem.*, 1939, v129, 547.

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Toxic Effect on the Chick Embryo of Homologous Tissue Suspensions Following Intravenous Inoculation. (17941)

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In attempting to adapt a neurotropic virus to chick embryos by intravenous passage the initial injection of a 10% infected mouse brain suspension produced no abnormal reaction. However, when 10% infected chick embryo brain suspension was similarly inoculated the embryos died within a few hours. Upon examination, multiple hemorrhages were noted in the skin and viscera. Brain suspensions of normal chick embryos were found to produce the same lesions. Similar phenomena in chick embryos have been briefly mentioned by Eichhorn(1) and Beveridge and Burnet(2). It has long been known that the intravenous inoculation of homologous and heterologous tissue extracts may be followed by sudden death or less severe reactions (3). In certain instances these effects appeared to be dependent upon the intravascular clotting of the blood. An example of such phenomena is afforded by the observations of Thomas(4,5). In mice injected with suspensions of normal mouse brain, an immediate reaction occurred consisting of ataxia, convulsions and coma. Administration of heparin prevented the reaction. Thomas attributed the phenomenon to the clotting action of thromboplastin. Green and Stoner(6) also recently analyzed the factors underlying the immediate lethal response of rats to intravenous inoculation of homologous tissue extracts. They concluded that it depends upon the combined action of thromboplastin and adenosine triphosphate and/or related compounds.

As far as we are aware, differences in the capacity of the extracts of tissue from various classes of animals to induce such reactions *in vivo* have not hitherto been clearly defined, although *in vitro* differences in thromboplastins have frequently been described. Accordingly, the phenomenon we have encountered has been further investigated.

Preparation of tissue suspensions. Suspensions used for injection were prepared by grinding the tissues in a mortar with a small amount of alundum. Sufficient isotonic buffer solution (pH 7.2) was added to make a 10% suspension based on the wet weight of the tissue. This material was centrifuged at 1500 r.p.m. for 15 minutes in an International Centrifuge fitted with a No. 2 head. The opalescent supernatant fluid was employed for injection. As a rule the suspensions were prepared on the day that they were used.

Technic of inoculation. Chick embryos were inoculated intravenously by the method of Eichhorn(1). Mice were injected into the tail vein, and hens into the wing vein.

Test for thromboplastin activity. Blood, with precautions to minimize trauma, was obtained in a syringe containing enough of a solution of 1% sodium oxalate in isotonic saline to give a final concentration of 0.1%. The plasma was separated by centrifugation. To 5 drops of plasma were added 3 drops of 0.5% calcium chloride and 2 drops of either tissue suspension or commercial thromboplastin prepared from beef tissue. The tubes were

* Post-doctorate Fellow, United States Public Health Service, present address L'Institut Pasteur, Paris, France.

† Senior Research Fellow in Virus Diseases of the National Research Council.

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2. Beveridge, W. I. B., and Burnet, F. M., The Cultivation of Viruses and Rickettsiae in the Chick Embryo, Med. Res. Council, Spec. Rep. Series No. 256, His Majesty's Stationery Office, London, 1946, p. 23.

3. Wooldridge, L. C., *Arch f. Physiol.,* 1886, p. 397.

4. Thomas, L., *Bull. Johns Hopkins Hosp.,* 1947, v81, 1.

5. Thomas, L., *Bull. Johns Hopkins Hosp.,* 1947, v81, 26.

6. Green, H. N., and Stoner, H. B., *Brit. J. Exp. Path.,* 1947, v28, 189.

TABLE I.
Effect of Intravenous Injection of Chick Embryo Brain Suspension Into 14-day-old Chick Embryos.

	Dilution of tissue suspension				
	1:10*	1:20	1:40	1:80	1:160
Mortality	4/4†	4/4	3/5	0/5	1/5
Hemorrhages‡	+	+	+	—	0

* Dilution based on wet weight of tissues.
† Numerator represents number of embryos dying, denominator the number injected.
‡ Plus indicates that multiple hemorrhages were observed upon inspection of the intact embryo after death.

agitated frequently and clotting determined by tilting the tube.

Effect of the injection of homologous tissue suspensions on the chick embryo and adult chicken. In 11- to 14-day-old chick embryos death with multiple hemorrhages regularly followed the intravenous injection of various quantities of brain suspension prepared from 13-day-old normal chick embryos. The data of one experiment are given in Table I. Several other experiments with brain suspensions prepared from 11- to 14-day-old embryos gave essentially the same results as well as preparations from the brains of newly hatched chicks and adult chickens. Suspensions of the following tissues induced the same response: Whole embryo (5 days); whole embryo without brain (13 days); chorioallantoic membrane; yolk sac. In accordance with the findings of Eichhorn(1), normal allantoic fluid from 12-day embryos caused death upon intravenous inoculation into 13-day embryos. Whether or not this effect depended upon the same factor as was demonstrated in the materials just enumerated is rendered doubtful by the absence of definite hemorrhage.

The following materials proved innocuous: egg yolk (diluted 1:2); defibrinated chick embryo blood (diluted 1:10); amniotic fluid. It is evident, therefore, that the active principle is present in many tissues, although it is not universally distributed.

In distinction to the findings in embryos 11- to 14-day-old, no hemorrhages were seen following the inoculation of comparable tissue suspensions into 18-day embryos, although death regularly ensued. Instead large clots were observed in the heart. Intravascular clots, in the absence of hemorrhage were also found in a hen that died two minutes after the intravenous inoculation of about 3 cc of a 20% chick embryo brain suspension.

Histologic examination of embryos of different ages after inoculation of 20% chick brain suspension revealed changes compatible with the findings in the gross. Interstitial hemorrhages and vascular congestion without evident deposition of fibrin were observed in embryos ranging from 9 to 15 days in age. In 18-day embryos and in the hen interstitial hemorrhages were not seen. Marked vascular congestion, however, with fibrin deposits in the large vessels and heart were observed.‡

Effect of injection of mouse tissue extract in the chick embryo and adult chicken. Twenty per cent suspensions of normal mouse brain, capable of reproducing the phenomenon described by Thomas(4) in mice were without effect when introduced intravenously into chick embryos. In addition suspensions of mouse liver, lung and spleen proved inactive. Comparable suspensions of mouse embryonic brain and of whole mouse embryo produced no toxic effect in chick embryos, nor were any symptoms noted after intravenous inoculation of about 3 cc of 20% mouse brain suspension into an adult chicken.

Effect of chick embryonic tissues in the mouse. Conversely, no effect was produced in mice by the intravenous injection of suspensions of various chick embryonic tissues (whole embryos, brain, chorioallantoic membrane, yolk sac).

Effect of various agents upon the toxicity of tissue suspensions. Heat. The activity of chick embryonic brain suspensions was not reduced by heating at 55° for 30 minutes, but was completely destroyed by heating at 65° for the same period. *Heparin.* It was hoped that the use of heparin would afford a practical means of neutralizing the activity of chick embryonic material and thus permit the injection of homologous tissue in chick embryos by the intravenous route. It was found

‡ We are very grateful to Dr. Orville Bailey for the preparation of the material for histologic examination and for his aid in interpreting the lesions observed.

TABLE II.
Effect of Heparin on Toxicity of Chick Embryo Brain Suspension.

	Dilution of heparin*						
	1:20	1:40	1:80	1:160	1:200	1:320	No heparin
Deaths†	0/3	1/3	2/3	3/3	3/3	2/3	3/3
Hemorrhages‡	—	+	++	+++	+++	+++	+++

* The stock heparin preparation used was Liquaemin (Roche-Organon) solution containing 10 mg/cc of sodium heparin. One volume of this solution in the dilutions indicated was mixed with one volume of 10% chick brain suspension. 0.05 cc of the mixture was inoculated intravenously into 13-day chick embryos.

† The numerator refers to the number of embryos that died, the denominator to the number injected.

‡ The number of hemorrhages visible upon inspection of the intact embryo after death was roughly estimated and graded from 0 = none to ++++ = many.

TABLE III.
Capacity of Tissue Suspensions from Various Species to Clot Human and Chicken Plasma.

Tissue suspension	Source of plasma		
	Adult chicken	Chick embryo	Man
Chick embryo brain	+	+	0 or ±
Adult chicken brain	+	+	0 or ±
Mouse brain	0 or ±	0 or ±	+
Beef lung*	0 or ±	0 or ±	+

* Desiccated beef lung (Upjohn).

that in sufficient concentration heparin, mixed with an appropriate quantity of homologous brain suspension, prevented multiple hemorrhage in the chick embryo. (Table II). However, following the injection of heparin, either alone or with tissue suspensions, massive hemorrhage often followed at the site of inoculation resulting in rapid death. For example 1 mg of heparin was inoculated into each of 14 embryos (12 days). Hemorrhage at the point of inoculation and death occurred in 8. Death occurred in only one case after the intravenous inoculation of 14 embryos with isotonic buffer solution. *Homologous and heterologous sera.* Thomas(5) reported that the toxic effect for mice or rabbits of mouse tissue suspensions could be neutralized by incubation with normal rabbit serum. The reaction took place only when calcium was present in the system. Using his technic, various quantities of chick embryo brain suspension were incubated with normal rabbit serum, normal mouse serum and with the serum of a normal adult chicken. When these mixtures were injected into chick embryos and the results compared with those obtained with the same tissue suspensions incubated in the presence of isotonic salt solution, no evidence of an inhibitory effect of the sera was demonstrated.

Possible relationship between the toxic factor and thromboplastin. Specificity of thromboplastin. The pathologic changes induced by the homologous tissue suspensions suggested that the active principle was thromboplastin. Accordingly the capacities of tissue suspensions derived from the chicken, mouse and ox to clot human and chicken plasma *in vitro* were investigated. The results are summarized in Table III. Human plasma was employed in place of mouse plasma, since we were unsuccessful in obtaining specimens of the latter that did not either clot spontaneously or upon the addition of calcium alone. These findings indicate a specific difference between avian and mammalian thromboplastins. Moreover the identity of the toxic factor with thromboplastin is suggested by their parallel specificity.

This evidence for a difference in the specificity of thromboplastins supplements that provided by the early work of Delezenne(7), Loeb(8,9), Hewlett(10) and Nolf(11), all of whom demonstrated *in vitro* differences in

TABLE IV.
Clotting of Plasma from Chick Embryos of Varying Ages by 10% Suspension of Chick Embryonic Brain.

	Age in days of embryos yielding plasma			
	10	13 and 15	17	19
Characteristic of clot	slight ppt.	heavy ppt.	stringy clot	solid clot

thromboplastins derived from various species or classes of animals. These old observations, however, appear to have frequently been disregarded by modern writers on blood coagulation.

Removal of the toxic factor during the clotting of plasma by tissue extracts. Additional evidence for the close association of the toxic factor with thromboplastin was obtained in the following manner. A 10% chick embryonic brain suspension, shown to be toxic for chick embryos, was mixed with an equal volume of adult chicken plasma and the clotted plasma centrifuged. It was found that the supernatant fluid was not toxic for embryos and no toxic property could be extracted from the clot after grinding in isotonic buffer at pH 7.2.

Possibility that the toxic factor is distinct from thromboplastin. There is, therefore, much evidence to suggest the identification of the toxic factor with thromboplastin. The fact, however, that the blood of the chick embryo does not acquire the capacity to form a typical clot during the earlier stages of development, makes it impossible to assert that all the effects observed *in vitro* are the result of thromboplastic activity. That the blood of the embryo fails to clot or to clot typically before the latter phases of embryonic development was observed long ago(12,13,14). In experiments of our own, as indicated in Table IV, it was found that when homologous embryonic brain suspension and calcium chloride were added to oxalated plasma from 10-day chick embryos no true coagulum developed; however a fine, very scanty precipitate slowly formed. The plasma from 13- and 15-day embryos yielded a more voluminous precipitate. In that from 17-day embryos a definite but loose and stringy coagulum was generated. Only from 19-day embryos was plasma obtained which produced a firm clot comparable to that characteristic of adult chicken plasma.

If these phenomena observed *in vitro* occur also *in vivo* when chick embryos of varying ages are inoculated with homologous tissue suspensions, it is evident that the formation of large firm intravascular thrombi cannot account for the presence of multiple hemorrhages in the younger embryos, or their death. It is possible that finely dispersed particles of precipitate comparable to those formed *in vitro* in the plasma of young embryos may develop *in vivo* and cause capillary thrombosis, with subsequent formation of multiple small hemorrhages. But in the light of available evidence, it cannot be concluded that the characteristic changes in young embryos and their death are dependent on the intravascular deposition of fibrin.

Comment. Since we have already discussed certain of the theoretical aspects of the phenomena that have been investigated we shall here mention only their possible practical application. To a virologist desiring to employ the intravenous route in the serial passage of preparations of infected homologous tissues the rapid death of the chick embryo will present an obstacle that as

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14. Pickering, J. W., and Gladstone, R. J., *Proc. Roy. Soc. of London, Series B*, 1925, v98, 516.

far as we know now can be circumvented only by employing relatively high dilutions of such materials, or perhaps, in certain instances, by the simultaneous intravenous injection of heparin. In the preparation of tissue cultures where chicken plasma is used, mammalian tissues or tissue extracts cannot be relied upon to induce the formation of a firm coagulum. The results of experiments designed to determine the anaphylactogenic capacity of various tissues or organs might be misinterpreted if the investigator were unaware of the rapid death which may follow the intravenous injection of tissue suspensions from certain related classes of animals and careful post mortem examinations were not made.

Conclusion. The toxic effect *in vivo* of certain tissue derivatives exhibits biologic specificity. This specificity appears to parallel that of thromboplastin on the clotting of blood plasma *in vitro*.

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The Preparation and Biological Activity of Alpha Estradiol-Sensitized Collodion Particles.* (17942)

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In the course of studies on the antigenic properties of crystalline alpha estradiol† (in combination with protein substances) we included collodion particle agglutination(1) as one of the tests for the presence of serum antibody. This serological procedure has proved to be a sensitive and reliable method of antibody detection and titration in our laboratory(2), as well as in others(3,4,5). The usual method of sensitization of collodion particles consists of incubation of a mixture of particles and antigen in optimal proportions. Non-specific absorption of antigen occurs, and the sensitized particles may then be combined with antisera in proper dilutions. This method of sensitization was not successful when aqueous suspensions of alpha estradiol were incubated with the collodion particles. We were not able to overcome the insolubility of the steroid substance in the aqueous medium. However, sensitization of collodion particles has been accomplished by incorporation of the steroid in and on the particle at the time of preparation.

Preparation of sensitized particles. Collodion U. S. P. Merck was treated in the manner recommended by Cannon and Marshall (1). Either a 5 or 10% acetone solution of the dried collodion constituted the stock solution to be used as a source of particles. Arbitrary amounts of crystalline alpha estradiol were dissolved in 100 ml quantities of the stock solution. In most instances we added sufficient alpha estradiol to give 100 mg per 100 ml of the collodion solution. The particles were then prepared and washed in the usual manner. The alpha estradiol content of the final particle suspension was calculated by lyophilizing quantities of 1-5 ml, weighing of the dried material and comparison

* This work was supported by a grant-in-aid from the United States Public Health Service.

† We are indebted to the Schering Corporation for supplying the alpha estradiol used in these studies.

1. Cannon, P. R., and Marshall, C. E., *J. Immunol.*, 1940, v38, 365.

2. Weir, J. M., PROC. SOC. EXP. BIOL. AND MED., 1941, v46, 47.

3. Dulaney, Anna Dean, and House, Virginia, PROC. SOC. EXP. BIOL. AND MED., 1941, v48, 620.

4. Cavelti, P. A., and Cavelti, E. S., *Arch. Path.*, 1945, v39, 148.

5. Cavelti, P. A., PROC. SOC. EXP. BIOL. AND MED., 1945, v60, 379.

TABLE I.
Biological Activity of Alpha Estradiol-Sensitized Collodion Particles.

Animal	Dose, ml	Lot	Calculated concentration of alpha estradiol, γ	Duration of cornified vaginal smear
Spayed mice	.1	2	1	9 days
	.1	3	1	9 "
	.1	1	1.6	27 "
	.2	1	3.2	45 "*
	.3	1	4.8	45 "*
Spayed rats	1.	1 (diluted)	1	5 "
	1.	2 "	1	5 "
	1.	3 "	1	5 "
	1.	3 "	5	15 "
	1.	2	10	30 days to date†
Weanling rats	1.	1	10	‡

Spayed mice gave no reaction to non-sensitized collodion particles.
* Duration of experiment.
† Cornified smear persists.
‡ Vaginal diaphragm opened in 48 hr, vaginal smear cornified through test period, 40 days.

with the collodion content of the original solution.

Animal tests. Biological evidence that the collodion particles carried the alpha estradiol was furnished by introduction of graded amounts of particle suspension into spayed mice and rats and into weanling rats. Table I presents the results of these tests. The spayed animals exhibited prompt evidence of estrogenic stimulation as indicated by the predominance of cornified cells in the vaginal smears. Within 48 hours the vaginal diaphragm of the weanling rats had opened and the vaginal smear showed only cornified cells. Moreover, the state of estrogenic stimulation following single injections in both species of animals persisted for substantially longer periods than those observed by us with the use of similar doses in oil or aqueous mediums. The periods of stimulation were related to the quantity of sensitized particles introduced. With the doses estimated to contain 10 gamma the animals have continued to show the effects of the hormone for periods of 4-6 weeks.

Apparently the alpha estradiol-sensitized particles provide an *in vivo* depot from which the estrogen is slowly eluted. It is believed that this demonstration that collodion particles may act as carriers of estrogens through incorporation of the steroid into and on the surface of the particles may offer opportunities for broad use. Detailed studies on this problem are in progress. These studies will include titrations of biological effects, histological examinations of the *in vivo* reservoir and use of the spectrophotometer for more exact determination of the alpha estradiol content of the collodion particle preparation.

Furthermore it is believed that such a method of sensitization of collodion particles may have practical use in study of other steroid "antigens" which include steroid-protein conjugates and tissue extracts. These data, along with those from use of the alpha estradiol-sensitized particles will form a separate report.

We should also like to emphasize that this method of sensitization of particles need not be limited to steroid substances. It should be possible to use collodion particles for the local deposit of various therapeutic reagents which owe their maximum effect to maintenance of adequate concentrations over a prolonged period.

Summary. Crystalline alpha estradiol may be dissolved in acetone solutions of collodion and the steroid incorporated in or on the collodion particles, prepared in the usual manner. Such particles are biologically active as shown by the onset of a cornified vaginal smear in spayed or immature mice and rats. Furthermore, the hormone stimulation appears to persist for prolonged periods, presumably due to the slow elution of the alpha

estradiol from the collodion particle.

Addendum. The alpha estradiol-sensitized collodion particles were active after a storage period of four and one half months in the refrigerator. This was established by the onset of cornified cells in spayed rats.

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Preparation of a Water Emulsion of α-Tocopherylhydroquinone Suitable for Intravenous Administration in Animals.* (17943)

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The recent observation that α-tocopherylhydroquinone† has vitamin E activity(1) made it desirable to have a preparation that is suitable for intravenous administration. The difficulty of preparing the tocopherols and those of their esters which are oils, in a form suitable for enzyme studies and for bioassay by parenteral administration is well known. These difficulties are increased in the case of α-tocopherylhydroquinone, because of the ease with which this substance is oxidized to tocopherylquinone. Ames(2) recently suggested the use of bovine serum as a menstruum for tocopherol in investigations on enzyme systems, and Sobel and co-workers (3,3a) have used aqueous dispersions of certain of the fat soluble vitamins. Since vitamin K can form a stable colloidal solution when added to sugar solutions(4) several sugars were investigated for their possible solubilizing effect on tocopherylhydroquinone. The 2 solvents, ethanol and propylene glycol have been employed in bioassays of tocopherylhydroquinone in this laboratory in the form either of a 100% propylene glycol solution, or of a 10% ethanol - 90% propylene glycol mixture. Propylene glycol produced traumatic effects on the blood vessels when injected intravenously daily. However, since these solvents are suitable for the chemical preparation of the α-tocopherylhydroquinone, both were investigated for their possible utilization in a satisfactory water emulsion. It was hoped that emulsions containing relatively low concentrations of these solvents might be free from the objectionable properties of the solvents themselves. The emulsions were not sterilized; this lack of sterilization appeared to offer no objection to their use in rabbits. However, similar employment in man should not be attempted.

Methods. Preparation of tocopherylhydroquinone. Tocopherylhydroquinone was prepared by hydrogenation of tocopherylquinone in either ethanol or propylene glycol, by the method previously described(1). The amounts of tocopherylquinone were such that the final solution contained 200 mg tocopherylhydroquinone per cc of ethanol, or 100 mg per cc of propylene glycol. The tocopherylhydroquinone was assayed colorimetrically by a modification of the Emmerie-Engel reaction(1).

Preparation of emulsions. Usually 1 cc of the solution was mixed with 9 cc of water in glass stoppered test tubes by underlaying

* Aided by the Armour Fund for Research in Muscular Disease and by a grant from The Nutrition Foundation, Inc.

† The α-tocopherylhydroquinone used in these studies was prepared from α-tocopherol kindly supplied by Merck & Co., Rahway, N. J.

1. Mackenzie, Julia B., Rosenkrantz, H., Ulick, S., and Milhorat, A. T., *J. Biol. Chem.*, 1950, v183, 655.

2. Ames, S. R., and Risley, H. A., *Ann. New York Acad. Sci.*, 1949, v52, 149.

3. Sobel, A. E., Rossberg, A., Geduldig, R., Engel, E., West, M., and Kramer, B., *Fed. Proc.*, 1949, v8, 253.

3a. Sobel, A. E., Sherman, M., Lichtblau, J., Snow, S., and Kramer, B., *J. Nutr.*, 1948, v35, 225.

4. Frank, H. A., Hurwitz, A., and Seligman, A. M., *N. E. J. Med.*, 1939, v221, 975.

the water with the tocopherylhydroquinone solution and then shaking the tube for a few seconds. The amounts of tocopherylhydroquinone were determined at various intervals after formation of the emulsion. These determinations indicated both the rate of oxidation of the tocopherylhydroquinone and the stability of the emulsion.

Observations and discussion. Ethanol-water emulsions proved unsatisfactory, and attempts to stabilize them with sugars were unsuccessful since less than 10% of the tocopherylhydroquinone could be accounted for in the emulsions. Isotonic saline, likewise, was unsatisfactory. The oxidation of tocopherylhydroquinone proceeds more slowly in propylene glycol than in ethanol. Propylene glycol-water emulsions prepared as outlined above contained from 80% to 90% of the tocopherylhydroquinone that had been added. The remainder presumably was present in the white crust on the surface of the emulsion. After periods of about 48 hours, 75% of the added tocopherylhydroquinone could still be accounted for in the emulsion. The emulsion had a white, milky appearance and examination under the microscope revealed a homogenous arrangement of the globules. Filtration through No. 1 Whatman paper or centrifugation for periods up to ½ hour did not disturb the emulsion significantly. After the emulsion had stood at room temperature for more than 24 hours, oxidation of the tocopherylhydroquinone was evident by the appearance of the yellow color characteristic of the quinone. The rates of oxidation of tocopherylhydroquinone in the emulsion and in propylene glycol are compared in Fig. 1. Initial concentrations of the hydroquinone in the water emulsion and in propylene glycol were 6.81 mg/cc and 5.41 mg/cc, respectively. The preparations were kept at refrigerator temperatures except for the intervals when the concentrations of tocopherylhydroquinone were being determined. It can be seen from Fig. 1 that the rates of oxidation of tocopherylhydroquinone are practically identical in both systems. At refrigerator temperatures, only slight oxidation occurred during the first 3 days and the emulsion still contained 50% or more of the added tocopherylhydroquinone after a period of one week.

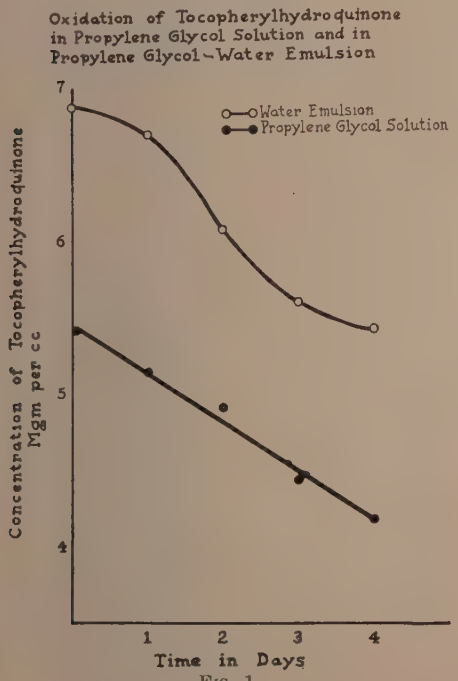

FIG. 1.

In order to ascertain if sugars could function as protective agents, emulsions composed of solutions of different sugars and tocopherylhydroquinone in propylene glycol were compared. The results are tabulated in Table I. Although the amounts of tocopherylhydroquinone that could be accounted for at zero time varied, these variations may not be significant and probably were the result of slight differences in the conditions permitting oxidation in the samples. However, there appeared to be no definite advantage in substituting solutions of sugars for distilled water in these emulsions.

Alterations in the concentration of either the tocopherylhydroquinone or the propylene glycol had adverse effects on the stability of the emulsion; maximal stability was achieved when the concentrations mentioned above were employed; namely, about 80 mg tocopherylhydroquinone in each 100 cc of a 10% propylene glycol-90% water emulsion. Tocopherol itself formed unsatisfactory propy-

TABLE I.
Effect of Certain Sugars and Other Substances on Formation and Stability of Propylene Glycol-Water Emulsion Containing Tocopherylhydroquinone.*

		THQ in emulsion†					
		At 0 time		At 24 hr		At 48 hr	
Substance added to water	THQ added to system mg/cc total vol.	mg/cc	% of added THQ	mg/cc	% of THQ at 0 time	mg/cc	% of THQ at 0 time
0‡	7.6	6.1	81	5.9	97	5.5	90
Inositol	7.6	6.5	86	5.9	91	5.4	83
Sorbitol	7.6	6.3	83	5.8	92	5.6	89
Arabinose	8.0	6.3	79	5.8	92		
Mannose	8.0	6.2	78	5.6	91	5.5	89
Galactose	8.0	6.2	78	5.7	92	5.0	81
Peptone	8.0	6.6	82	6.3	95	5.8	88
Glycine	8.0	6.4	80	6.0	94	5.3	83

* 1 cc propylene glycol containing tocopherylhydroquinone was added to 9 cc water or water containing the sugars or other substances as 1% solution.
† For purposes of brevity, tocopherylhydroquinone is designated as THQ.
‡ Control, water only.

TABLE II.
Effect of Salts on Propylene Glycol-Water Emulsion Containing Tocopherylhydroquinone.*

		THQ in emulsion†					
		At 0 time		At 1 hr		At 24 hr	
Salt	mg salt added	mg/cc	% of added THQ	mg/cc	% of THQ at 0 time	mg/cc	% of THQ at 0 time
	0‡	5.81	85	5.25	90	4.83	83.5
NaCl	9	5.72	84	5.25	92	3.01	52.5
$CaCl_2$	12.2	5.72	84	3.36	59	2.87	50
$MgSO_4 \cdot 7H_2O$	19.1	5.72	84	1.75	30.5		
KCl	11.5	5.72	84	5.18	90.5	3.15	55

* 1 cc propylene glycol containing tocopherylhydroquinone was added to 9 cc water or water containing the salts as shown.
† For purposes of brevity, tocopherylhydroquinone is designated as THQ.
‡ Control, water only.

lene glycol-water emulsions. Propylene glycol-water emulsions have been used routinely in this laboratory for the daily intravenous administration of tocopherylhydroquinone in rabbits. One rabbit received daily injections of the emulsion for 7 weeks without signs of irritation to the blood vessels(1). It would appear that such emulsions are useful for parenteral administration in animals. Since the media commonly employed in enzyme studies contain electrolytes, the effect of ions on the propylene glycol-water emulsion was studied. The concentrations used were identical with those employed in Krebs-Ringer Solution. The data given in Table II indicate that the addition of electrolytes results in early separation of the emulsion.

Summary. A relatively stable emulsion of α-tocopherylhydroquinone in propylene glycol and water can be formed. Best results were obtained when propylene glycol solutions containing 100 mg/cc of the hydroquinone were used and the concentration of propylene glycol in the emulsion was 10%. Such emulsions contained 90% of the added hydroquinone and were relatively stable. The emulsion was useful for intravenous administration in rabbits and produced no significant local reaction in the blood vessels. The emulsion has not been administered parenterally in patients; its injection by the intravenous route in man must await further investigation, and

the development of satisfactory methods of sterilization.

We wish to thank Mr. Stanley Ulick for suggestions, and Miss Maeda Mayran for technical assistance.

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Unusual Antigenic Variation in an Enteric Bacterium. (17944)

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The bacterium to be described is represented by 8 cultures, all of which were isolated by Hinshaw and McNeil(1) in their study of salmonellosis of reptiles. The 8 cultures were isolated from the viscera of as many fence lizards *(Sceloporus occidentalis)* captured along the banks of a stream in Davis, California. According to a personal communication from Dr. Hinshaw, the viscera of each animal were ground, placed in the tetrathionate broth of Kauffmann for enrichment and plated on brilliant green agar. Although the organism was isolated repeatedly from lizards captured at intervals in the same locality, there was no evidence that it produced disease. From the meager information available, this organism would seem to be commonly present in the lizards in that locality.

The bacterium was a motile rod which possessed the usual characteristics of the *Enterobacteriaceae.* It produced hydrogen sulfide, was methyl red positive and Voges-Proskauer negative, failed to form indol or to liquefy gelatin, and gave negative reactions in Stern's glycerol-fuchsin broth. The organism promptly produced acid from d-tartrate and mucate but did not attack l- or i-tartrate. Citrate was utilized as evidenced by growth on Simmons' medium but in fluid medium some of the cultures did not completely utilize the substance. Acid and gas were produced from glucose, arabinose, xylose, rhamnose, trehalose, maltose, sucrose, raffinose, sorbitol, dulcitol and mannitol within 24 hours. Cellobiose was fermented after 5 days. Lactose, inositol, and salicin were not attacked. The cultures possessed a well developed avidity for sucrose, after overnight incubation marked acidity and moderate gas formation were observed in sucrose broth. These characteristics indicate that the organism is an intermediate coliform bacterium which is not readily classifiable in any of the presently recognized genera of the *Enterobacteriaceae.* It was recognized by Hinshaw and McNeil that the cultures were agglutinated by O serum for group B *Salmonella* but no further observations concerning their antigenic characteristics were recorded. Upon examination it was found that the strains were agglutinated to the titre of group B serum and possessed antigens IV, XII_1, XII_2. Mirror absorption tests with serums prepared from *Salmonella abortus-equi* and from the lizard strains established the identity of the O antigens of the two types.

The first cultures received were poorly motile and continuous passage through semi-solid agar was necessary to maintain the cultures in a condition in which they were flocculated well by H serums. The reactions obtained with Pc 231 were typical of those of all the cultures studied and are recorded in Table I. It was found that prompt agglutination occurred in polyvalent *Salmonella* serum and in *Salmonella typhi* serum (d). In addition, the cultures were flocculated slowly and in low dilution in serum for phase 1 of *Salmonella reading* (e,h). Thus it seemed that the cultures possessed antigens d,e,h and that antigen d was the major component while

1. Hinshaw, W. R., and McNeil, E., *J. Bact.*, 1947, v53, 715.

TABLE I.
H Antigens of Pc231.

Serums	Antigens						
	S. typhi (d)	*S. reading* phase 1 (e,h)	*S. glostrup* phase 2 (e,n,z_{15})	Pc231 (d,e,h)	Pc231 (d)	Pc231 (e,h)	Pc231 (e,n,z_{15})
S. typhi (d)							
Unabsorbed	10000	0	0	5000	5000	0	0
Absorbed by							
Pc231 (d,e,h)	200			0	0		
Pc231 (d)	200			0	0		
S. reading (e,h)							
Unabsorbed	0	10000	500	500	0	10000	500
Absorbed by							
Pc231 (e,h)		0	0	0		0	0
S. glostrup (e,n,z_{15})							
Unabsorbed	0	500	10000	0	0	1000	10000
Absorbed by							
Pc231 (e,n,z_{15})		0	0			0	0
Pc231 (d,e,h)							
Unabsorbed	5000	200	0	5000	5000	200	0
Absorbed by							
Pc231 (d)	0	100		50	0	200	
S. typhi (d) +							
S. reading (e,h)	0	0		500	500	0	
S. oregon (d) +							
S. reading (e,h)	0	0		0	0	0	
Pc231 (e,h)							
Unabsorbed	0	5000	500	1000	0	10000	500
Absorbed by							
S. reading (e,h)		0	0	0		0	0
Pc231 (e,n,z_{15})							
Unabsorbed	0	1000	20000	0	0	2000	20000
Absorbed by							
S. glostrup (e,n,z_{15})		0	0			0	0
Pc231 (d)							
Unabsorbed	10000	0	0	20000	20000	0	0
Absorbed by							
S. typhi (d)	0			500	500		
S. oregon (d)	0			0	0		

antigens e,h were present in small amount. In order to determine whether this antigenic complex was separable under ordinary conditions of culture, the various strains were plated and numerous colonies examined. In all, more than 400 single colony cultures were studied. With one exception all the single colony isolations behaved as did the parent strains. One colony from Pc 219, the first culture studied, failed to flocculate in d serum but reacted strongly in e,h serum, as would a typical e,h phase. From these results it may be assumed that the lizard strains are monophasic, have the formula IV,XII : d,e,h in which the major H antigen is d and that rarely, under usual conditions of culture, variants occur which have the formula IV,XII : e,h and which appear to be stable.

To investigate more fully the variational potentialities of the cultures they were examined by a modification of the Gard technic devised by Edwards and Bruner(2). When the organisms were grown in semisolid agar with agglutinating serums, phases having antigens d; e,h; and e,n,z_{15} were isolated. The

2. Edwards, P. R., and Bruner, D. W., *Ky. Agric. Exp. Sta. Cir.* 54, 1942.

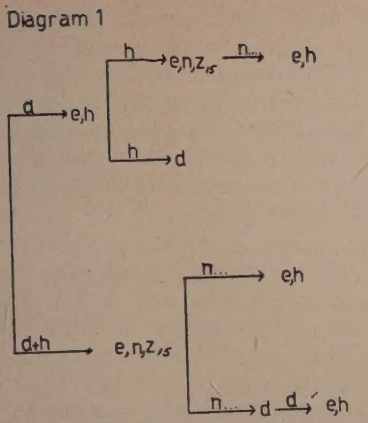

FIG. 1.

Symbols on arrows indicate serums in which cultures were grown.

d—*S. typhi* serum unabsorbed.

h—*S. reading* phase 1 serum absorbed by *S. abortus-equi*.

n . . .—*S. abortus-equi* serum absorbed by *S. reading*, phase 1.

variations observed in the cultures are summarized in Fig. 1. The results are a composite of those obtained with all the cultures but most of the changes shown were obtained with each strain. Pure d phases were obtained only from two cultures, but e,h and e,n,z_{15} phases were obtained from all. The experiments were done with carefully selected single colony cultures and each time antigenic change occurred the cultures were plated and single colonies selected for subsequent work.

When the original cultures (d,e,h) were placed in e,h serums, the organisms spread slowly through the medium and the antigenic characters of the spreading growth were unchanged. Apparently the e,h component was so slightly developed that the corresponding serum had no immobilizing effect. When the cultures were placed in d serum, they were immobilized. After one or 2 days incubation filmy bulbs appeared which spread through the medium and this spreading growth was composed of typical e,h phases. If the original cultures were placed in both d and e,h serums no evidence of antigenic change was seen until the cultures were transferred 2 or 3 times, when bulbs composed of e,n,z_{15} phases appeared. Subsequent changes were accomplished with even greater difficulty; often the organisms were carried through long series of transfers in serum mediums before evidence of change was noted.

The salient characters of the d,e,h; d; e,h; and e,n,z_{15} phases are given in Table I. For the sake of brevity only a few of the many absorption tests performed are included. It is evident that the d antigen of the lizard strains, like the d antigens of many *Salmonella* types, is not identical with the d of *S. typhi*. *Salmonella oregon* removed all d agglutinins from serums prepared from the d phase of the lizard strains but reciprocal tests not included in the table demonstrated that the lizard cultures did not have the entire d complex of *S. oregon*. An interesting observation was that the d antigen of the IV,XII : d,e,h cultures was identical with the d of a sucrose fermenting lizard culture described by Edwards, Moran and Bruner(3) which had antigens VI,VIII : d,i. The pure d phases were identical with the d antigen of the d,e,h phases, as shown by suitable absorption tests.

The e,h and e,n,z_{15} phases of the lizard strains were identical with phase 1 of *S. reading* and phase 2 of *Salmonella glostrup* respectively. So far as could be determined, the phases obtained by growth in serums were stable under usual conditions of culture. No changes were observed in them during a 2 year period and serums prepared from them contained no minor components for other antigens.

The cultures described here are similar in many ways to *Salmonella salinatis* (IV,XII : d,e,h-d,e,n,z_{15}) of Edwards and Bruner(4). They differ from the latter form in that they ferment sucrose and are monophasic. Further, it was never possible to demonstrate the natural occurrence of loss variants in *S. salinatis* nor could antigen d ever be recovered from e,h or e,n,z_{15} phases induced by growth in serum. Stable d phases were not found in *S. salinatis*. The question naturally occurs as to whether the lizard cultures should be

3. Edwards, P. R., Moran, A. B., and Bruner, D. W., *J. Bact.*, 1948, v60, 529.

4. Edwards, P. R., and Bruner, D. W., *J. Bact.*, 1942, v44, 289.

placed in the genus *Salmonella* or whether *S. salinatis* should be removed from the genus. Against the latter view is the fact that it easily is possible to induce *Salmonella sandiego* (IV,XII : e,h-e,n,z_{15}), a commonly occurring *Salmonella* type, from *S. salinatis*. Although they differ in biochemical properties, the IV,XII : e,h variants which appear naturally in the lizard strains are antigenically identical with the monophasic *Salmonella* forms described by Cherry, Barnes and Edwards(5). The classification of cultures such as those described here is an arbitrary matter and must be the subject of international agreement.

The isolation of three stable phases from one culture is most unusual and has not previously been reported. From these phases it was possible to prepare three excellent diagnostic serums for *Salmonella* typing. The fact that no monophasic *Salmonella* possessing antigens e,n,z_{15} has yet been found makes that form of the lizard strains quite useful and it has been used for routine production of typing serum.

Summary. Eight sucrose fermenting cultures isolated from lizards were found to have the antigenic formula IV,XII : d,e,h. From them it was possible to obtain forms having the formulas IV,XII : d; IV,XII : e,h; and IV,XII : e,n,z_{15}. The IV,XII : e,h form occurred spontaneously as a loss variant under the usual conditions of culture. The variants were quite stable and could be used for the production of diagnostic serums.

5. Cherry, W. B., Barnes, L. A., and Edwards, P. R., *J. Bact.*, 1946, v51, 235.

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